Express Mail Label No.: EV 717191793 US
Date of Deposit: April 3, 2009

DOCKET NO.: G0744.90026US00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Paul DiTullio et al.
Patent No.:	6,441,145
Confirmation No.:	3148
Issued:	August 27, 2002
For:	TRANSGENICALLY PRODUCED ANTITHROMBIN III
Examiner:	D. Crouch
Art Unit:	1632

MAIL STOP HATCH-WAXMAN PTE
Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RECEIVED
APR 0 3 2009
PATENT EXTENSION
OPLA

Sir:

APPLICATION FOR PATENT TERM EXTENSION UNDER 35 U.S.C. § 156

Applicant, GTC Biotherapeutics, Inc., hereby submits this application for patent term extension under 35 U.S.C. § 156 of Patent No. 6,441,145 ("the '145 patent") in light of the FDA's approval of Biologic License Application (BLA) 125284 on February 6, 2009. Pursuant to 37 C.F.R. § 1.740(b), this application is accompanied by the required additional two (2) copies, for a total of three (3) copies.

Applicant is the assignee of the entire right, title, and interest in the '145 patent by virtue of an assignment recorded at Reel 007592, Frame 0745. Applicant was formerly named "Genzyme Transgenics Corporation", and the change of name to GTC Biotherapeutics, Inc. was recorded at Reel 013782, Frame 0450. BLA 125284 was approved in the name of GTC Biotherapeutics, Inc.

For the convenience of the PTO and the FDA, this application will address the requirements for an application for patent term extension in the order presented in 37 C.F.R. § 1.740(a).

04/21/2009 KLOGAN 00000001 09143195
01 FC:1457 1120.00 OP

1. The approved product, ATryn (Antithrombin (Recombinant)) is a recombinant human antithrombin. Antithrombin (Recombinant) is a 432 amino acid glycoprotein with a molecular

weight of approximately 57,215 Daltons. The molecular formula is $C_{2191}H_{3457}N_{583}O_{656}S_{18}$. Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk.

The amino acid sequence of Antithrombin (Recombinant) is identical to that of human plasma-derived antithrombin. Antithrombin (Recombinant) and plasma-derived antithrombin both contain six cysteine residues forming three disulphide bridges and 3-4 N-linked carbohydrate moieties. The glycosylation profile of Antithrombin (Recombinant) is different from plasma-derived antithrombin, which results in an increased heparin affinity.

2. ATryn was subject to regulatory review under § 505 of the Federal Food, Drug, and Cosmetic Act and § 351 of the Public Health Service Act.

3. ATryn received permission for commercial marketing or use under § 351 of the Public Health Service Act on February 6, 2009.

4. The only active ingredient in ATryn is Antithrombin (Recombinant), which is approved for the prevention of peri-operative and peri-partum thromboembolic events in hereditary antithrombin deficient patients. Antithrombin (Recombinant) had not been approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act; the Public Health Service Act; or the Virus-Serum-Toxin Act before the approval of BL 125284 on February 6, 2009.

5. This application is being submitted within the 60-day period permitted by 37 C.F.R. § 1.720(f), the last day of which is April 6, 2009.

6. The complete identification of the patent for which a patent term extension is sought is as follows:

Inventors:	Paul DiTullio; Harry Meade; Edward S. Cole
Patent Number:	6,441,145
Issue Date:	August 27, 2002

Expiration Date: February 21, 2015

7. A copy of the '145 patent is attached. (Attachment A)

8. The '145 patent was terminally disclaimed. A copy of the terminal disclaimer is attached. (Attachment B) No Certificate of Correction or Re-examination Certificate has been issued. Attached is a copy of the receipt verifying payment of the 4th year maintenance fee in 2006. (Attachment C) The 8th year maintenance fee payment window is not yet open.

9. Claims 1-6 and 11-14 of the '145 patent cover ATryn, and claims 7-9, 15-16, and 18-19 cover methods of producing ATryn. As demonstrated in the attached claim chart (Attachment D), ATryn is a mammary gland produced antithrombin III having a monosaccharide composition which comprises GalNAc, as specified by product claim 1. As also demonstrated in the attached claim chart, ATryn is produced in mammalian milk by a. providing a transgenic mammal that expresses in its mammary tissue a transgene which encodes a human antithrombin III with a monosaccharide composition which comprises GalNAc, wherein said human antithrombin III is secreted into the milk of the mammal; and b. collecting milk from the transgenic animal which contains the human antithrombin III; c. to thereby obtain human antithrombin III with a monosaccharide composition which includes GalNAc, as specified by method of manufacturing claim 7.

10. The relevant dates and information pursuant to 35 U.S.C. § 156 (g) to enable the Secretary of Health & Human Services to determine the applicable regulatory review period are as follows:

Investigational New Drug (IND) Application IND 6881 for ATryn was submitted on October 15, 1996 and became effective on November 14, 1996.

IND 10941 for ATryn was submitted on February 23, 2003 and became effective on April 1, 2003.

BLA 125284 for ATryn was submitted on January 31, 2008 and became effective on September 30, 2008.

BLA 125284 for ATryn was approved on February 6, 2009.

11. As a brief description of the significant activities undertaken by applicant during the regulatory review period, attached are three chronologies (IND 6881, IND 10941 and BLA 125284) of the significant communications of substance between the applicant and the FDA. (Attachment G)

12. Applicant is of the opinion that the '145 patent is eligible for an extension under 35 U.S.C. § 156 because it satisfies all the requirements for such an extension as follows:

(a) 35 U.S.C. § 156 (a) – The '145 patent includes claims covering Antithrombin (Recombinant), the active ingredient of ATryn, and methods for producing Antithrombin (Recombinant).

(b) 35 U.S.C. § 156 (a)(1) – The '145 patent has not expired before submission of this application.

(c) 35 U.S.C. § 156 (a)(2) – The term of the '145 patent has never been extended under 35 U.S.C. § 156 (e)(1).

(d) 35 U.S.C. § 156 (a)(3) – This application is submitted by the owner of record of the patent in accordance with the requirements of paragraphs (1) through (4) of 35 U.S.C. § 156 (d) and the applicable rules of the PTO.

(e) 35 U.S.C. § 156 (a)(4) – ATryn has been subject to a regulatory review period before its commercial marketing or use.

(f) 35 U.S.C. § 156 (a)(5)

(A) – Except as provided in subparagraph (B) or (C), the permission for the commercial marketing or use of the ATryn (Antithrombin (Recombinant)) after such regulatory review period is the first permitted commercial marketing or use under the provisions of § 505 the Federal Food, Drug, and Cosmetic Act or § 351 of the Public Health Service Act.

(B) – Alternatively, the '145 patent claims a method of manufacturing ATryn (Antithrombin (Recombinant)) which primarily uses recombinant DNA technology, and the permission for the commercial marketing or use

of ATryn after such regulatory period is the first permitted commercial marketing or use of a product manufactured under the process claimed in the '145 patent.

(g) 35 U.S.C. § 156 (c)(4) – No other patent has been extended for the same regulatory review period for ATryn.

The length of the extension of patent term of the '145 patent claimed by applicant is 1243 days (three (3) years, four (4) months and twenty-seven (27) days), resulting in an expiration date of July 18, 2018. The length of the extension was determined pursuant to 37 C.F.R. § 1.778 as follows:

(a) The Regulatory Review Period under 35 U.S.C. § 156 (g)(1)(B) began on November 14, 1996 and ended on February 6, 2009, for a total of 4469 days, which is the sum of (1) and (2) below.

(1) The period of review under 35 U.S.C. § 156 (g)(1)(B)(i) ("the Testing Period"), began on November 14, 1996 and ended on September 30, 2008, which is 4339 days.

(2) The period of review under 35 U.S.C. § 156 (g)(1)(B)(ii) (the "Approval Period") began on September 30, 2008 and ended on February 6, 2009, which is 130 days.

(b) The Regulatory Review Period upon which the period of extension is calculated is the entire Regulatory Review Period determined above (4469 days) minus:

(1) The number of days in the regulatory review period which were on or before the date on which the patent issued (August 27, 2002), 35 U.S.C. § 156(c), which is 2113 days;

(2) The number of days during which applicant or its agent did not act with due diligence, 35 U.S.C. § 156(c)(1), which is zero (0) days; and

(3) One-half of the Testing Period after the '145 patent issued (one half of 2226 days), *see* 35 U.S.C. § 156(c)(2), which is 1113 days.

The resulting number of days is 1243 days. Adding 1243 days to the original expiration date of the '145 patent (February 21, 2015) results in the extended expiration date of July 18, 2018.

(c) The term from FDA approval until expiration of the extended patent term cannot exceed fourteen (14) years. 35 U.S.C. § 156(c)(3). Fourteen (14) years when added to the date of the BLA approval of February 6, 2009 would result in the date of February 6, 2023. The extended patent expiration date of July 18, 2018 therefore does not violate the 14-year limit.

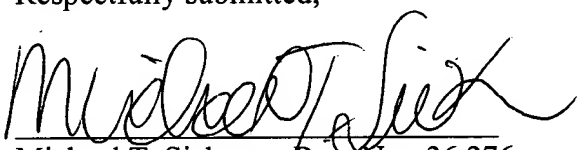
(d) Since the patent issued after September 24, 1984, the period of extension may not exceed five (5) years. 35 U.S.C. § 156(g)(6)(A). Five (5) years when added to the original expiration date of the patent (February 21, 2015) would result in the date of February 21, 2020. The extended patent expiration date of July 18, 2018 therefore does not violate the 5-year limit.

13. Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Human and Health Services any information that is material to the determination of entitlement to the extension sought.

14. The prescribed fee for receiving and acting upon this application is provided in the amount of $1,120.00. The Director is authorized to charge any additional fees required by this application to Deposit Account No. 23/2825.

15. All correspondence and inquiries may be directed to the undersigned, whose address, telephone number, and fax number are provided below.

Respectfully submitted,

By: 

Michael T. Siekman, Reg. No.: 36,276
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000
Facsimile: (617) 646-8646

Docket No.: G0744.90026US00
Date: April 3, 2009

Attachments:

Attachment A - Copy of '145 patent
Attachment B - Terminal disclaimer
Attachment C - Receipt verifying maintenance fee payment
Attachment D - Showing that each applicable patent claim reads on ATryn or methods of producing ATryn with
Attachment E - Package Insert
Attachment F - Cited *Blood* Reference
Attachment G - Three chronologies of significant communications with FDA

US006441145B1

(12) **United States Patent**
DiTullio et al.

(10) **Patent No.: US 6,441,145 B1**
(45) **Date of Patent: *Aug. 27, 2002**

(54) **TRANSGENICALLY PRODUCED ANTITHROMBIN III**

(75) Inventors: **Paul DiTullio**, Framingham; **Harry Meade**, Newton; **Edward S. Cole**, Mendon, all of MA (US)

(73) Assignee: **Genzyme Transgenics, Inc.**, Framingham, MA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/143,155**

(22) Filed: **Aug. 28, 1998**

Related U.S. Application Data

(63) Continuation of application No. 08/391,743, filed on Feb. 21, 1995, now Pat. No. 5,843,705.

(51) **Int. Cl.**[7] **C07K 1/00**; C07K 14/00; C07K 16/00; C07K 17/00; C12P 21/00

(52) **U.S. Cl.** **530/393**; 530/380; 530/381; 530/386; 530/392; 530/395; 800/7

(58) **Field of Search** 800/7, 21, 14, 800/18; 536/24.5; 514/2–21; 435/69.1; 530/380, 381, 386, 392, 393, 395, 832; 930/240

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,517,294 A	5/1985	Bock et al.	435/70
4,632,981 A	12/1986	Bock et al.	530/393
4,873,316 A	10/1989	Meade et al.	530/412
5,366,894 A	11/1994	Clark et al.	435/320.1

OTHER PUBLICATIONS

Mullin et al., "Perspectives Series: Molecular Medicine in Genetically Engineered Animals, Transgenesis in the Rat and Larger Mammals." J. Clin. Invest. , vol. 98, No. 11, Suppl., S37–S40, 1996.*

Drohan WN, "The Past, Present and Future of Transgenic Bioreactors." Thrombosis and Haemostasis, vol. 78 (1): 543–547, 1997.*

Cole et al., "Glycoslyation Patterns of Human Proteins Expressed in Transgenic Goat Milk." Journal of Cellular Biochemistry, Supp. 0 (18D) p. 265, 1994,*

Edmunds et al., "Tissue specific and species differences in the glycosylation pattern of antithrombin III." Journal of Cellular Biochemistry Suppl., vol. 0 (18D) p. 265, 1994.*

Denman et al., "Transgenic expression of a variant of human tissue–type Plasminogen activator in goat milk: purification and characterization of the recombinant . . . " Biotechnology, 9:839–843, 1991.

Ebert et al., "Transgenic production of a variant of human tissue–type Plasminogen activator in goat milk: generation of transgenic goats and analysis . . . " Biotechnology, 9:836–838, 1991.

Bock, S. et al., "Cloning and Expression of the cDNA for Human Antithrombin III", *Nucleic Acids Research,* vol. 10 (24), PP. 8113–8125 (1982).

Cole, E. et al., "Glycosylation Patterns of Human Proteins Expressed in Transgenic Goat Milk",), pp. *I of Cellular Biochemistry,* Suppl. 0 (180D), pp. 265 (1994).

Edmunds, T. et al., "Tissue Specific and Species Differences in the Glycosylation Pattern of Antithrombin III", *Journal of Cellular Biochemistry,* Supp. 0 (180D), pp. 265 (1994).

Fan, B. et al., "Heterogeneity of Recombinant Human Antithrombin III Expressed in Baby Hamster Kidney Cells" *The Journal of Biological Chemistry,* vol. 268 (23), pp. 17588–17596 (1993).

Wall, R., "Transgenic Livestock: Progress and Prospects for the Future", *Theriogenology,* vol. 45, pp. 57–68 (1996).

* cited by examiner

Primary Examiner—Deborah Crouch
(74) *Attorney, Agent, or Firm*—Fish & Richardson, P.C.

(57) **ABSTRACT**

This invention relates to transgenically produced human Antithrombin III (tgATIII). The human ATIII produced by the transgenic process of the present invention has a monosaccharide composition which comprises N-acetylgalactosamine (GalNAc) along with fucose, N-acetylglucosamine, galactose, mannose, and N-acetylneuraminic acid/N-glycolyneuraminic acid. The monosaccharide composition differs with that of plasma derived ATIII (phATIII). It has been found that tgATIII has an increased clearance rate when compared to phATIII.

20 Claims, 11 Drawing Sheets

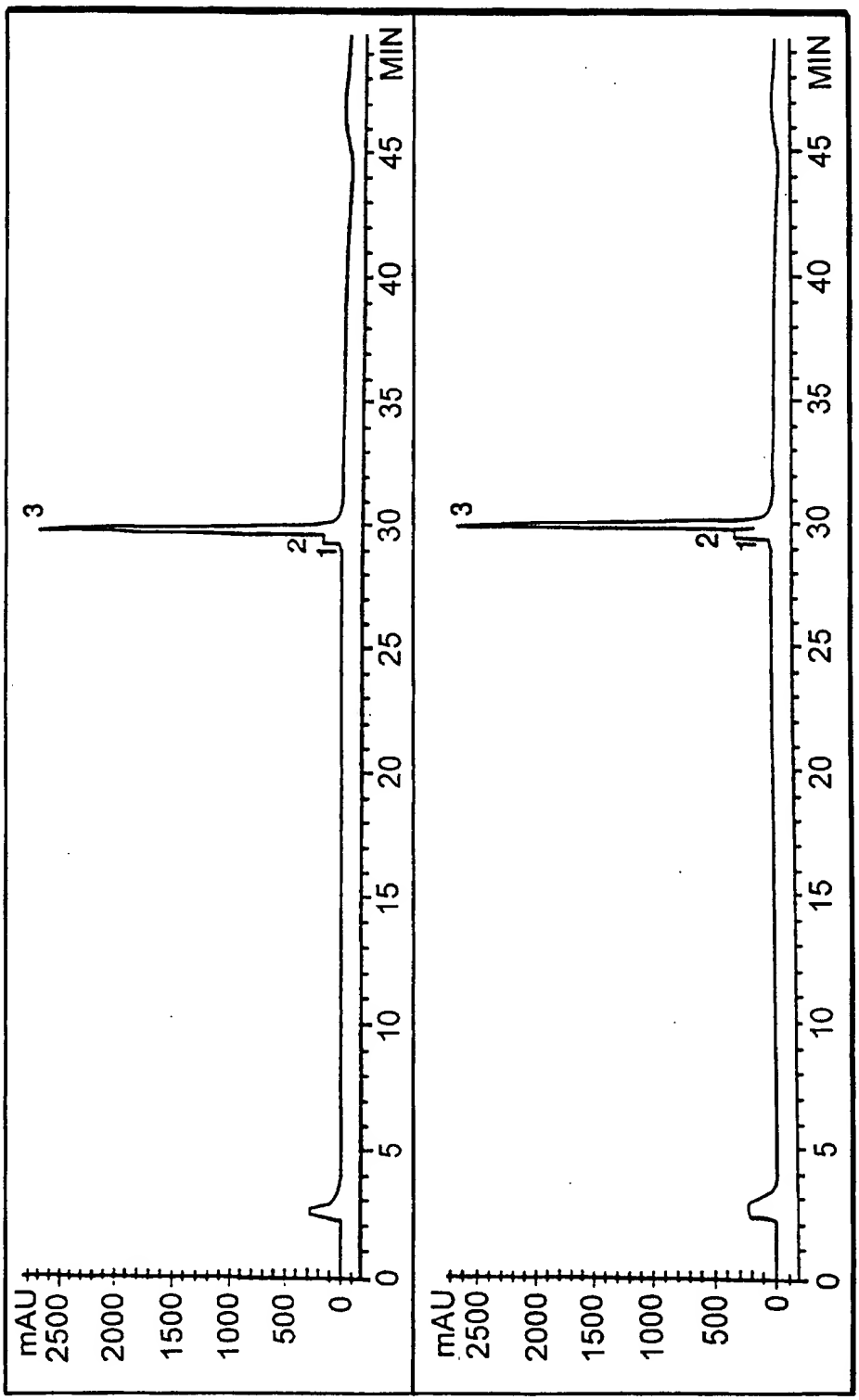


FIG. 1

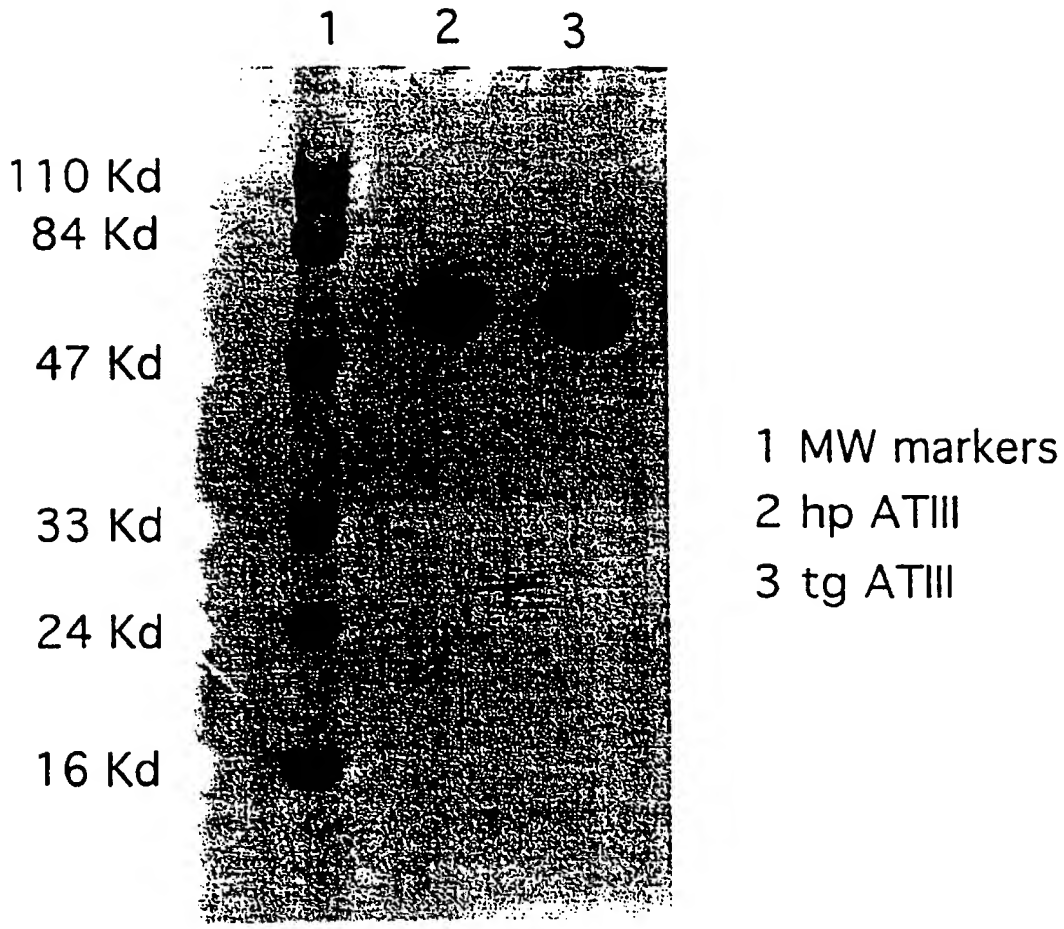

1
2
3
110 Kd
84 Kd
47 Kd
33 Kd
24 Kd
16 Kd
1 MW markers
2 hp ATIII
3 tg ATIII

FIG. 2

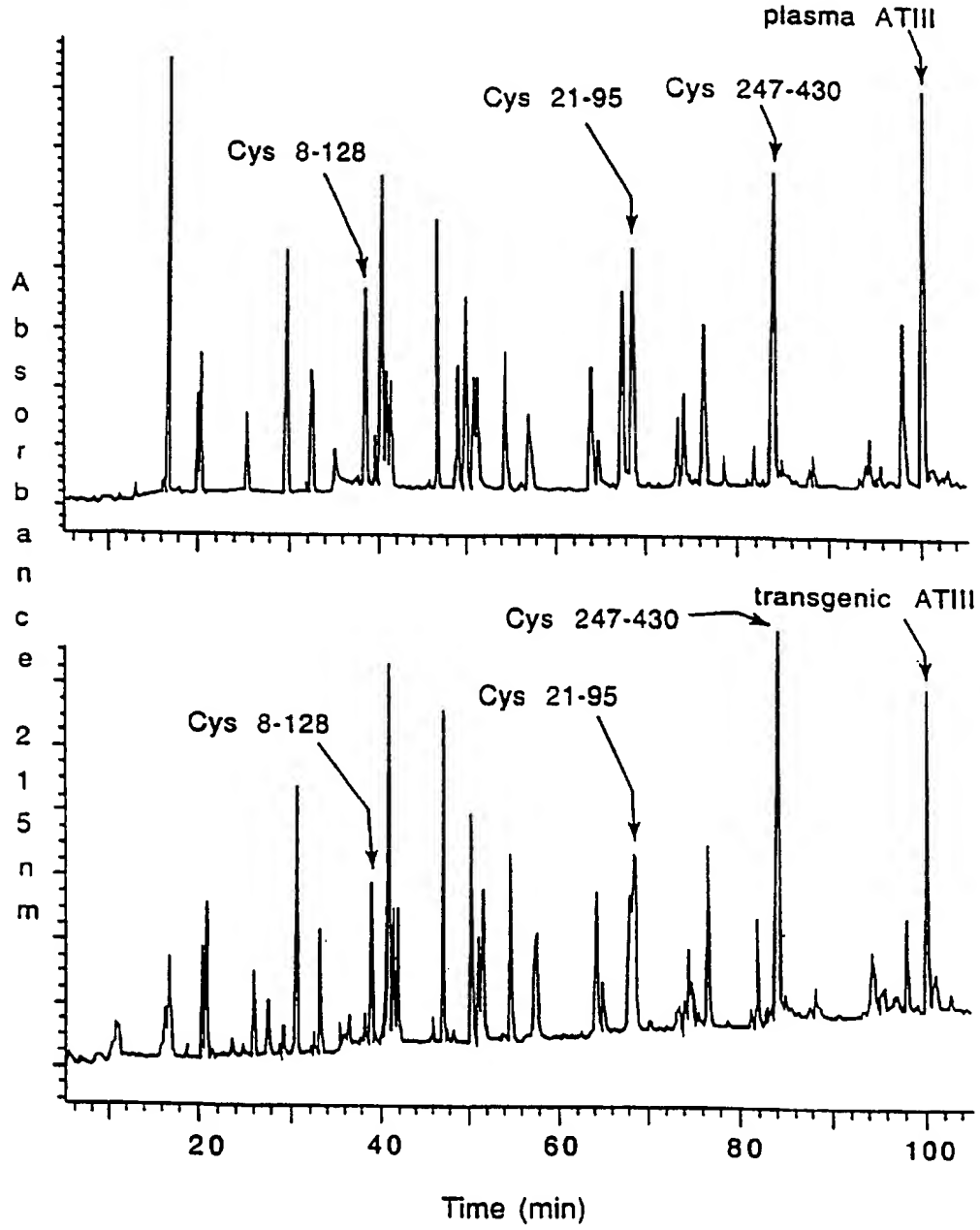

plasma ATIII
Cys 21-95
Cys 247-430
Cys 8-128
Absorbance 215 nm
transgenic ATIII
Cys 247-430
Cys 21-95
Cys 8-128
20
40
60
80
100
Time (min)

FIG. 3

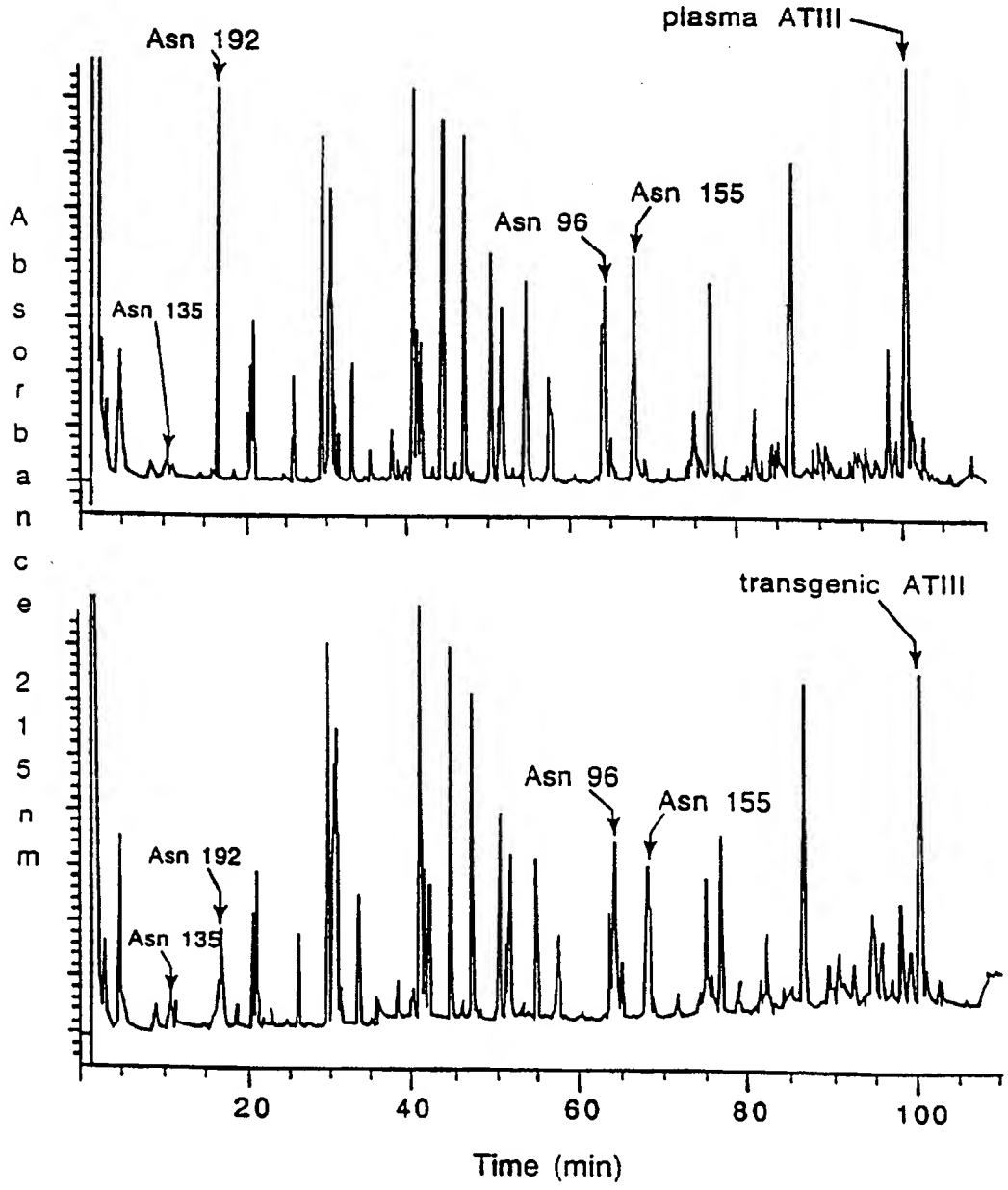

plasma ATIII
Asn 192
Asn 155
Asn 96
Asn 135
transgenic ATIII
Asn 96
Asn 155
Asn 192
Asn 135
Absorbance 215 nm
20
40
60
80
100
Time (min)

FIG. 4

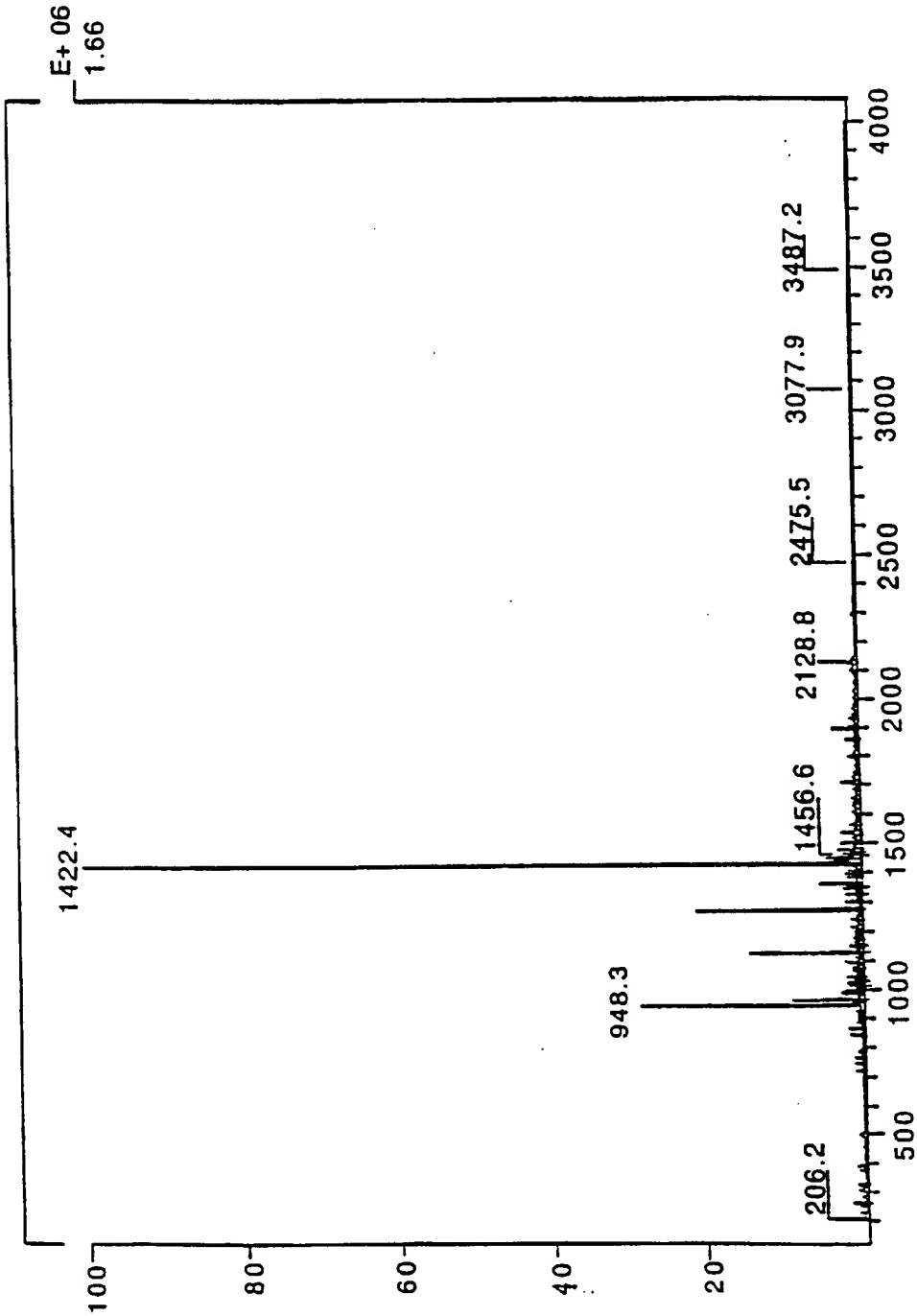

E+ 06
1.66
100
80
60
40
20
500
1000
1500
2000
2500
3000
3500
4000
206.2
948.3
1422.4
1456.6
2128.8
2475.5
3077.9
3487.2

FIG. 5

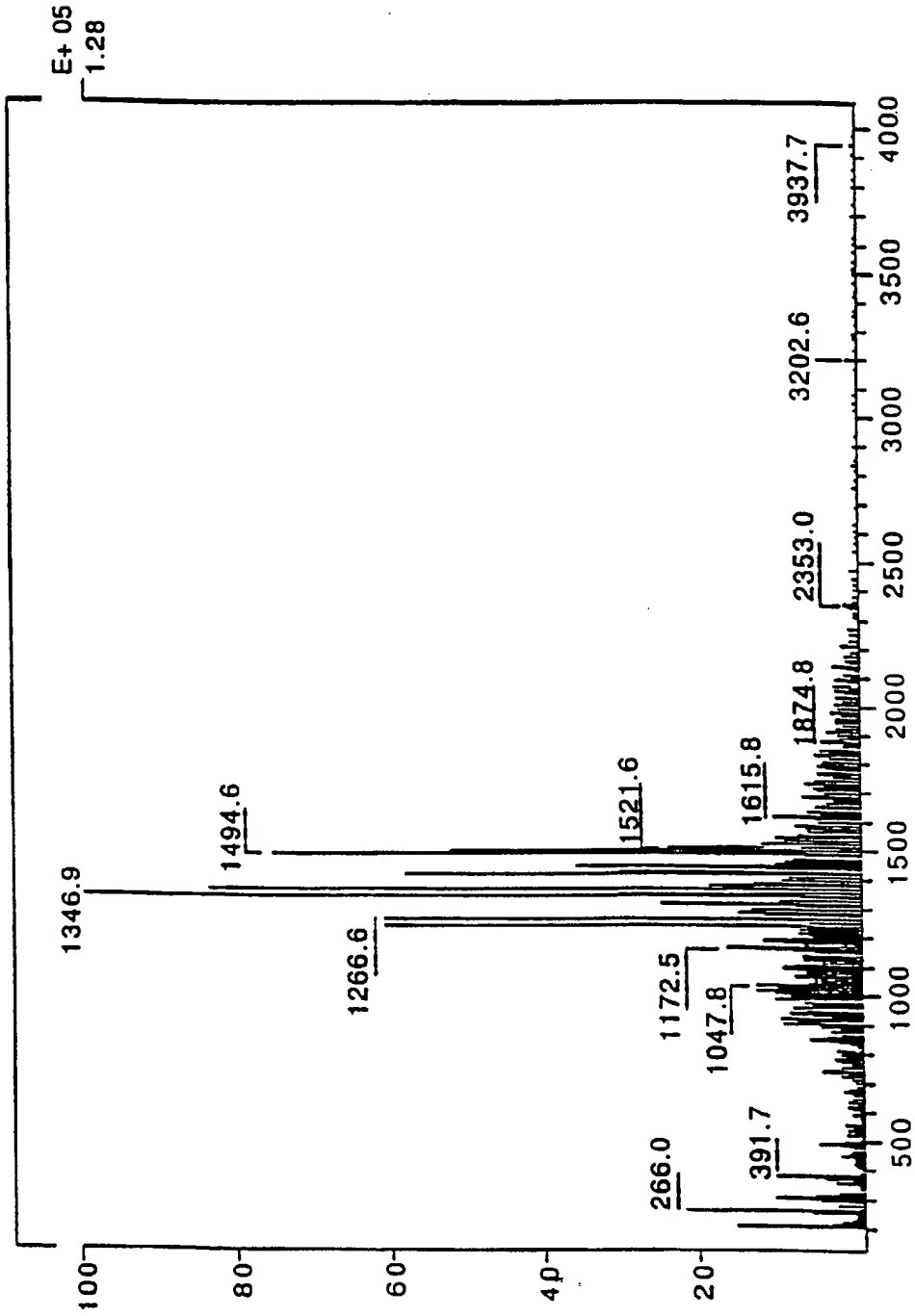

E+ 05
1.28
100
80
60
40
20
500
1000
1500
2000
2500
3000
3500
4000
266.0
391.7
1047.8
1172.5
1266.6
1346.9
1494.6
1521.6
1615.8
1874.8
2353.0
3202.6
3937.7

FIG. 6

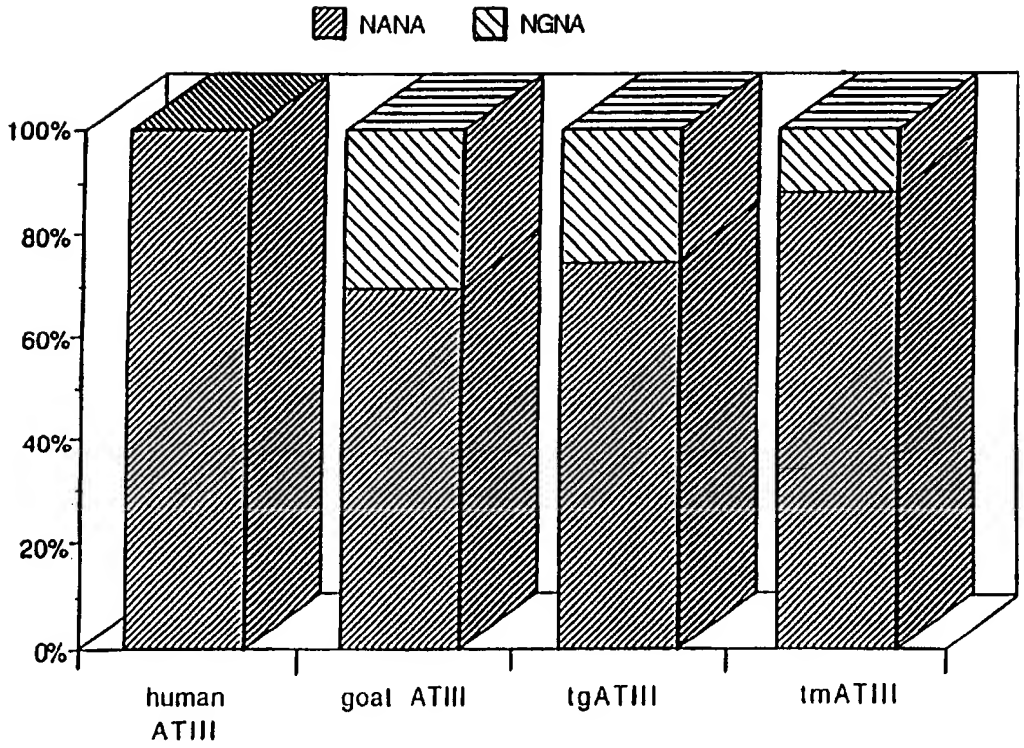

NANA
NGNA
100%
80%
60%
40%
20%
0%
human ATIII
goat ATIII
tgATIII
tmATIII

FIG. 7

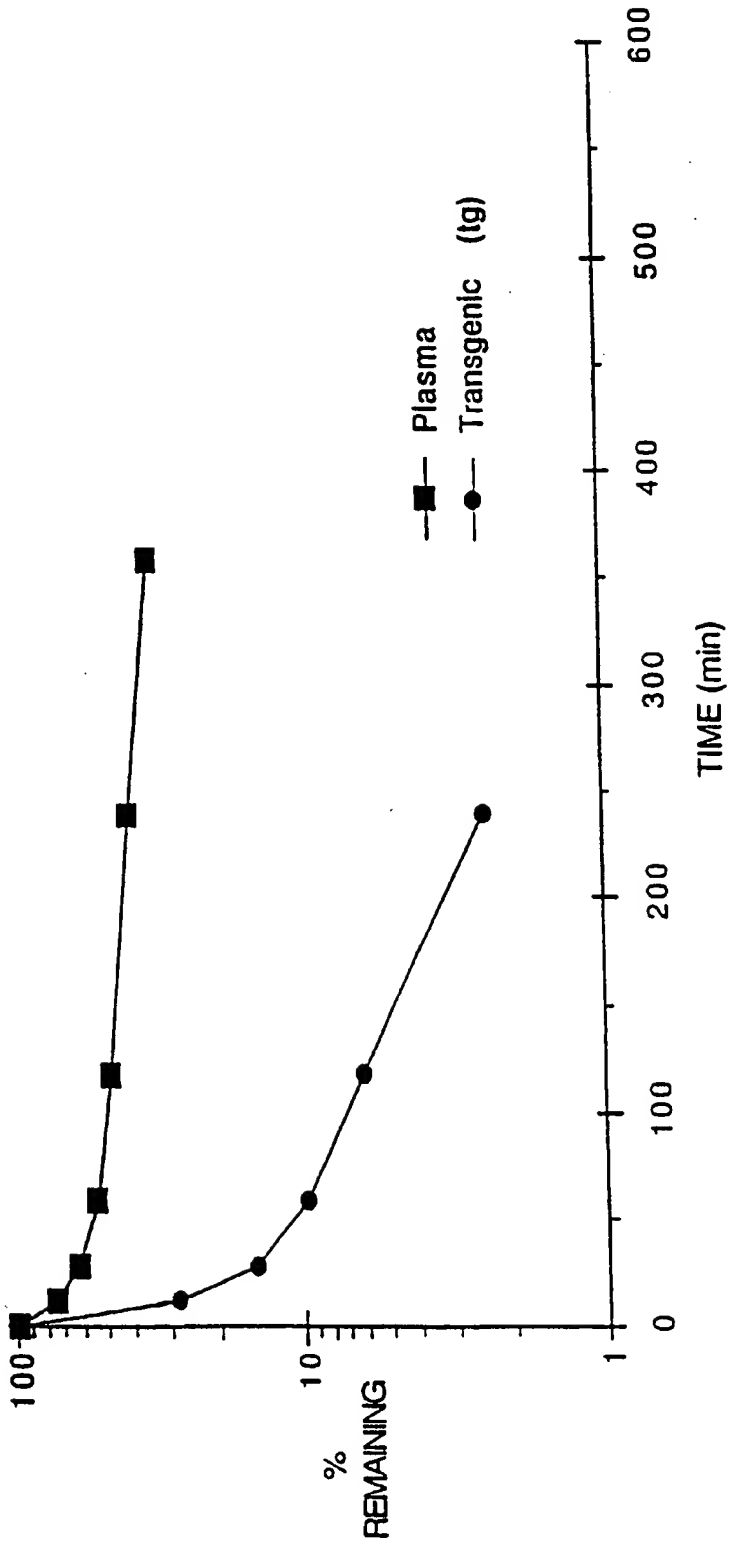

Plasma
Transgenic (tg)
100
10
1
% REMAINING
0
100
200
300
400
500
600
TIME (min)

FIG. 8

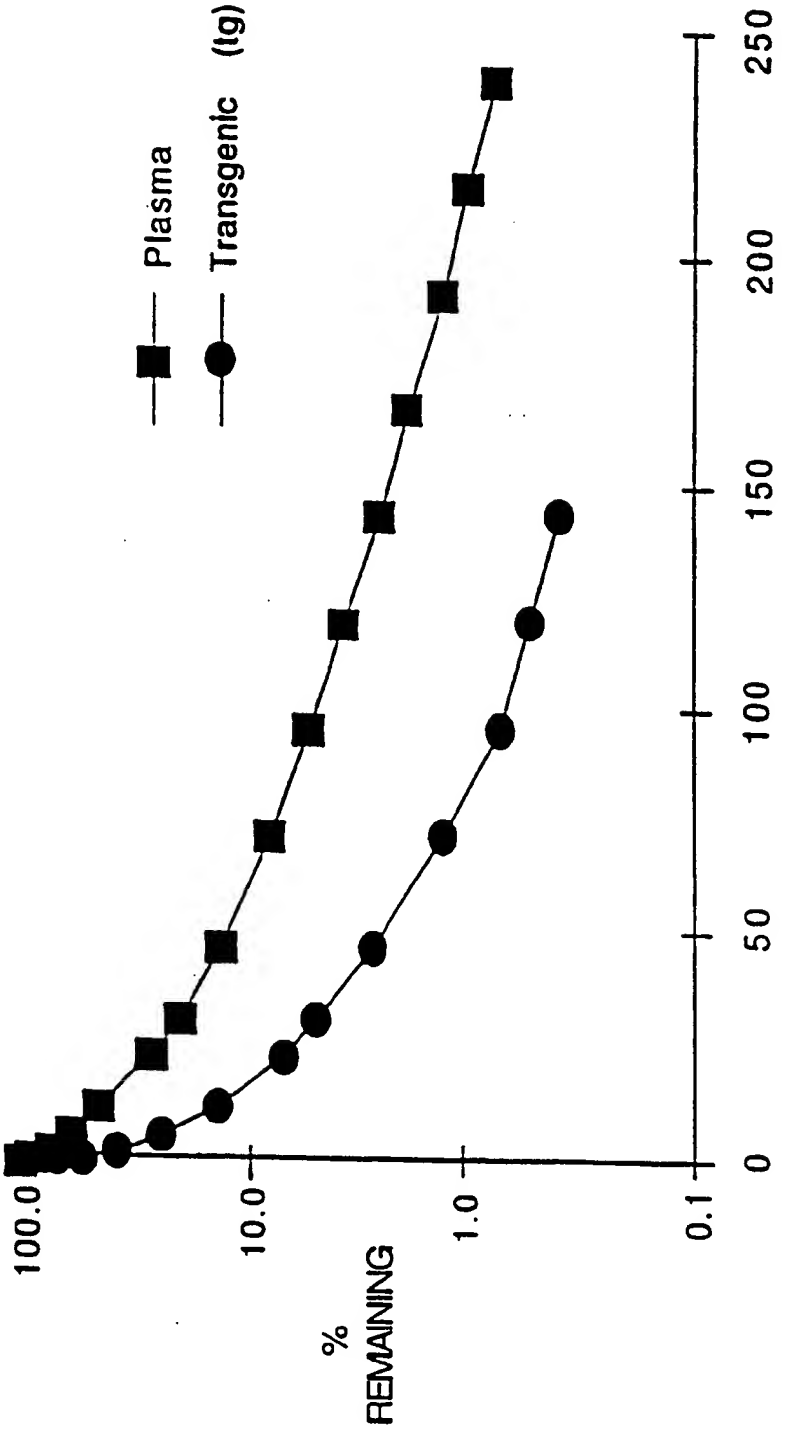

Plasma
Transgenic (Ig)
100.0
10.0
1.0
0.1
% REMAINING
0
50
100
150
200
250
HOURS

FIG. 9

Sequence Listing

```
agaatttaccccaagatctcaaagacccactgaatactaaagagacctcattgtggttac      -263
aataatttggggactgggccaaaacttccgtgcatcccagccaagatctgtagctactgg      -203
acaatttcatttcctttatcagattgtgagttattcctgttaaaatgctccccagaattt      -143
ctggggacagaaaaataggaagaattcatttcctaatcatgcagatttctaggaattcaa       -83
atccactgttggttttatttcaaaccacaaaattagcatgccattaaatactatatataa       -23
acagccactaaatcagatcattATCCATTCAGCTTCTCCTTCACTTCTTCTCCTCTACTT        37
TGGAAAAAAGgtaagaatctcagatataatttcagtgtatctgctactcatctttatttt        97
ggactaggttaaaatgtagaaagaacataattgcttaaaatagatcttaaaaataagggt       157
gtttaagataaggtttacactattttcagcagatatgttaaaaaatagaagtgactataa       217
agacttgataaaaattataggtgactgcaa.....ttttgccat                       277
gaggtttgcaggatcttggttccctgaccagggatcaaacctgcactcccctggaagcat       337
ggagtcttggacatttgtattatacactatctttggttccttttaaagggaagtaatttt       397
acttaaataagaaaatagattgacaagtaatacgctgtttcctcatcttcccattcacag       457
GAATCGAGAGCCATGAAGGTCCTCATCCTTGCCTGTCTGGTGGCTCTGGCCATTGCAAGA       517
   ****     M K V L I L A C L V A L A I A R     16
GAGgtaaatacagaaaaaatgttgaaataatagactagtactgtctgcctatgtgtagaa       577
E                                                                  17
atcacattaccaacatcataaatgtataaataatgcacaatctcagatttatttttaat       637
gctaagaaagtcatttatgttcatccactatctcaacagtatcctataggaccacaactc       697
tgggtcaagtgctttctatagtattgtaccatctgtaccatcaattcctaaagaaaaagg       757
aaaagaaaccaataagcaacagaccaacaagaaggaacacagacaagaacaaaaaatgag       817
taatattgtacaaatacaattgcacg.....ctgcaggaactaaa                      877
gtgtttttttttctctctcttttagCAGGAAGAACTCAATGTAGTCGGTGAGgtaagat       937
                         Q E E L N V V G E                        26
atttttatacaaagaaaaaaattaatttaactgtaaaatagtaacagactctgatgatct       997
agcagaaaactcagctaattgtcaatttttatttttccttttatagACTGTGGAAAGCCTT     1057
                                           T V E S L               31
TCAAGCAGTGAGgtaagataatgttcattcagaggcaatttcccagatttagagcaataa      1117
S S S E                                                            35
aacactgtattatctttttgtgttacattaattggcaacccactccagtactctt.....      1177
.............gacaaatatgaatttttctttaaagctaaacctgattttatttttt      1237
atttttccaaagGAATCTATTACACACATCAATAAGgtaaaaccttcatatttaaacgta      1297
          E S I T H I N K                                          43
cattttaaaaatttcatgtttgatttttataaacagcatttctttatgtgtgatttttt      1357
ttttaccagAAAATTGAGAAGTTTCAAAGTGAGGAACAACAGCAAACAGAGgtaatttg      1417
         K I E K F Q S E E Q Q Q T E                               57
ttcactatgagtatattttgagaagtattatgaaacataacacataaaagatttataata      1477
attatgttcagtctaagaatggtaatataagtgtcagtgtaagaaatgaaaactttgaca      1537
```

FIG. 10A

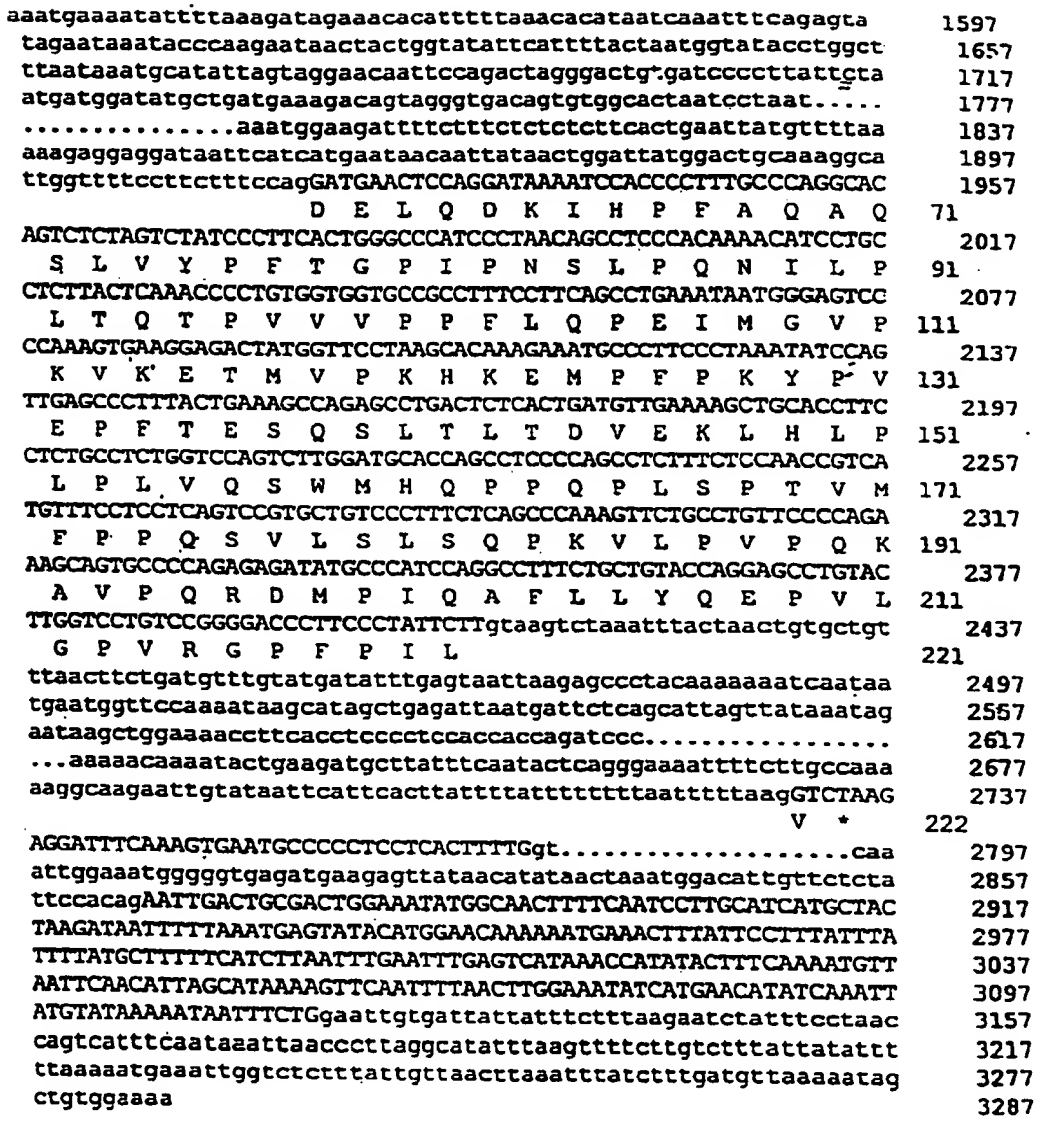

aaatgaaaatattttaaagatagaaacacatttttaaacacataatcaaatttcagagta 1597
tagaataaatacccaagaataactactggtatattcattttactaatggtatacctggct 1657
ttaataaatgcatattagtaggaacaattccagactagggactg gatccccttattcta 1717
atgatggatatgctgatgaaagacagtagggtgacagtgtggcactaatcctaat..... 1777
.....aaatggaagattttctttctctctcttcactgaattatgttttaa 1837
aaagaggaggataattcatcatgaataacaattataactggattatggactgcaaaggca 1897
ttggttttccttctttccagGATGAACTCCAGGATAAAATCCACCCCTTTGCCCAGGCAC 1957
D E L Q D K I H P F A Q A Q 71
AGTCTCTAGTCTATCCCTTCACTGGGCCCATCCCTAACAGCCTCCCACAAAACATCCTGC 2017
S L V Y P F T G P I P N S L P Q N I L P 91
CTCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAATAATGGGAGTCC 2077
L T Q T P V V V P P F L Q P E I M G V P 111
CCAAAGTGAAGGAGACTATGGTTCCTAAGCACAAAGAAATGCCCTTCCCTAAATATCCAG 2137
K V K E T M V P K H K E M P F P K Y P V 131
TTGAGCCCTTTACTGAAAGCCAGAGCCTGACTCTCACTGATGTTGAAAAGCTGCACCTTC 2197
E P F T E S Q S L T L T D V E K L H L P 151
CTCTGCCTCTGGTCCAGTCTTGGATGCACCAGCCTCCCCAGCCTCTTTCTCCAACCGTCA 2257
L P L V Q S W M H Q P P Q P L S P T V M 171
TGTTTCCTCCTCAGTCCGTGCTGTCCCTTTCTCAGCCCAAAGTTCTGCCTGTTCCCCAGA 2317
F P P Q S V L S L S Q P K V L P V P Q K 191
AAGCAGTGCCCCAGAGAGATATGCCCATCCAGGCCTTTCTGCTGTACCAGGAGCCTGTAC 2377
A V P Q R D M P I Q A F L L Y Q E P V L 211
TTGGTCCTGTCCGGGGACCCTTCCCTATTCTTgtaagtctaaatttactaactgtgctgt 2437
G P V R G P F P I L 221
ttaacttctgatgtttgtatgatatttgagtaattaagagccctacaaaaaaatcaataa 2497
tgaatggttccaaaataagcatagctgagattaatgattctcagcattagttataaatag 2557
aataagctggaaaaccttcacctcccctccaccaccagatccc..... 2617
...aaaaacaaaatactgaagatgcttatttcaatactcagggaaaatttttcttgccaaa 2677
aaggcaagaattgtataattcattcacttattttatttttttaatttttaagGTCTAAG 2737
V * 222
AGGATTTCAAAGTGAATGCCCCCTCCTCACTTTTGgt.....caa 2797
attggaaatgggggtgagatgaagagttataacatataactaaatggacattgttctcta 2857
ttccacagAATTGACTGCGACTGGAAATATGGCAACTTTTCAATCCTTGCATCATGCTAC 2917
TAAGATAATTTTTAAATGAGTATACATGGAACAAAAAATGAAACTTTATTCCTTTATTTA 2977
TTTTATGCTTTTTCATCTTAATTTGAATTTGAGTCATAAACCATATACTTTCAAAATGTT 3037
AATTCAACATTAGCATAAAAGTTCAATTTTAACTTGGAAATATCATGAACATATCAAATT 3097
ATGTATAAAAATAATTTCTGgaattgtgattattatttctttaagaatctatttcctaac 3157
cagtcatttcaataaattaacccttaggcatatttaagttttcttgtctttattatattt 3217
ttaaaaatgaaattggtctctttattgttaacttaaatttatctttgatgttaaaaatag 3277
ctgtggaaaa 3287

FIG. 10B

TRANSGENICALLY PRODUCED ANTITHROMBIN III

This application is a continuation of U.S. Ser. No. 08/391,743, filed Feb. 21, 1995, now U.S. Pat. No. 5,843,705, the contents of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

A growing number of recombinant proteins are being developed for therapeutic and diagnostic applications; however, many of these proteins may be difficult or expensive to produce in a functional form in the required quantities using conventional methods. Conventional methods involve inserting the gene responsible for the production of a particular protein into host cells such as bacteria, yeast, or mammalian cells, and then growing the cells in culture media. The cultured cells then synthesize the desired protein. Traditional bacteria or yeast systems may be unable to produce many complex proteins in a functional form. While mammalian cells can reproduce complex proteins, they are generally difficult and expensive to grow, and produce only mg/L quantities of protein.

The application of transgenic technology to the commercial production of recombinant proteins in the milk of transgenic animals offers significant advantages over traditional methods of protein production. These advantages include a reduction in the total amount of required capital expenditures, elimination of the need for capital commitment to build facilities early in the product development life cycle, and lower direct production cost per unit for complex proteins. Of key importance is the likelihood that, for certain complex proteins, transgenic production may represent the only technologically and economically feasible method of commercial production.

Antithrombin III (ATIII) is a serine protease inhibitor which inhibits thrombin and the activated forms of factors X, VII, IX, XI, and XII. It is normally present in serum at levels of 14–20 mg/dL. Decreased levels of ATIII may be found in the serum of individuals who have either a hereditary deficiency of ATIII or an acquired deficiency, which can result from a number of pathologic conditions. The conventional treatment for hereditary ATIII deficiency is protein replacement therapy, which may also be effective in treating some acquired deficiencies.

Current methods of obtaining ATIII involves isolating the protease inhibitor from blood plasma. However, the use of plasma-based ATIII presents various problems due to the many components in plasma, including: variation between lots; immunogenicity problems; and biohazardous risks due to viral contamination.

A need exists to develope a method to produce ATIII without the inherent problems of the present process.

SUMMARY OF THE INVENTION

This invention relates to transgenically produced human Antithrombin III (tgATIII). The human ATIII (hATIII) produced by the transgenic process of the present invention has a monosaccharide composition which comprises N-acetylgalactosamine (GalNAc) along with fucose, N-acetylglucosamine, galactose, mannose, and N-acetyineuraminic acid/N-glycolyneuraminic acid. The monosaccharide composition differs with that of human plasma derived ATIII (phATIII). It has been found that tgATIII has an increased clearance rate when compared to phATIII.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a chromatograph comparing the HPLC profile of the tgATIII of the present invention and plasma derived ATIII.

FIG. **2** is a photograph of a SDS page gel showing a greater than 98% purity of tgATIII.

FIG. **3** illustrates that the disulfide cross-linking between phATIII and the tgATIII. The peaks labelled with the arrows are the peaks containing disulfide cross-linked peptides.

FIG. **4** shows the individual equivalent peptides of tgATIII and phATIII by mass spectrometry.

FIG. **5** illustrates the mass spectrometry data for one of the individual glycopeptide residues from plasma derived ATIII.

FIG. **6** shows the mass spectrometry data for the same glycopeptide residue as shown for FIG. **5**, but for tgATIII.

FIG. **7** illustrates the sialic acid composition of human ATIII, goat ATIII, tgATIII, and transgenic mouse ATIII (tmATIII).

FIG. **8** shows a graph comparing the clearance rate of phATIII and tgATIII in mice.

FIG. **9** shows a graph comparing the clearance rate of phATIII and tgATIII in monkey.

FIGS. **10***a* and **10***b* depict the nucleotide sequence and the corresponding amino acid sequence of human antithrombin III.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based upon the discovery that hATIII produced by the transgenic method of the present invention is structurally different than ATIII extracted from plasma (phATIII). The monosaccharide composition of the transgenically produced ATIII (tgATIII) differs in the composition and the quantity when compared to plasma derived ATIII. In particular, the lycpsylation sites on the tgATIII vary considerably in contrast to phATIII which are all uniform.

Exemplification

Generation of the Gene Construct

A mammary gland-specific transgene was constructed by inserting the human Antithrombin III (hATIII) cDNA into the caprine beta casein gene (CSN2). The caprine beta casein gene was cloned as an 18.5 Kb fragment in a lambda EMBL3 vector (Roberts, et al., Gene., 1992. 121: p. 255–262). The 6.2 Kb promoter (including exon 1 and part of exon 2) was fused to the hATIII cDNA to direct high level mammary gland-specific expression. A 7.2 Kb 3' flanking region (including part of exon 7, exon 8, and exon 9) was added to the 3' end of the hATIII cDNA to help stabilize the expression levels. The 14.95 Kb transgene was excised from bacterial sequences and injected into goat embryos for the production of hATIII in goats' milk.

Identification of Gene Coding for the Protein of Interest

The hATIII cDNA was received from Dr G. ZettimeißI (Behringwerke A. G., Marburg, Germany) in the plasmid pBAT6. The sequence of the cDNA is the same as that published by Bock, et al., Nucleic Acids Research, 1982. 10: p. 8113–8125, except for the silent nucleotide changes at bp 1096 (T-C) and bp 1417 (A-G).

Identification of Regulatory Sequences of Interest

To direct high level tissue-specific expression of hATIII to the mammary gland of transgenic goats, the goat betascasein gene was cloned from a lambda EMBL3 goat genomic

library. The goat beta casein gene is a mammary gland-specific gene which directs expression of high levels of beta casein into the milk. In goats, beta casein is thought to comprise 25–50% of the total milk proteins (~10–20 mg/ml). The goat beta casein gene was cloned from a Saanen goat genomic library and characterized in transgenic mice as described in Roberts, et al., 1992.

Cloning DNA Fragments

High molecular weight goat DNA was isolated from a Saanen goat blood sample according to the procedure described by Herrmann and Frischauf, Methods Enzymology, 1987. 152: p. 180–183. The genomic DNA was partially digested with Mbol and ligated into BamHI cleaved lambda EMBL3 phage arms (Stratagene, La Jolla, Calif.) by standard methods (Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press, 1989). The Saanen goat genomic library consisted of 1.2×10^6 recombinant phage and was used to isolate the goat beta casein gene. The goat genomic library was screened using a 1.5 Kb HindIII/TthIII1 fragment encoding the entire mouse CSN2 cDNA (Yoshimura, et al., Nucleic Acids Res., 1986. 14: p. 8224) and three overlapping clones were identified. The three overlapping clones were designated EMBL 3–7, 3–8, and 3–11. Only clone 3–11 contained the entire goat beta casein gene and was used for all future analysis and vector construction (Roberts, et al., 1992).

Characterization of Cloned Material

The goat beta casein gene was isolated as an 18.5 Kb fragment in lambda EMBL3 phage clone designated EMBL3–11 (Roberts, et al., 1992). The fragment contains 4.2 Kb of 5' flanking region, exons 1 through 9, and 5.3 Kb of 3' flanking sequence. The gene was subcloned into pUC19 or pGEM3Z vectors. The subclones were sequenced by Sanger dideoxy sequencing to verify all intron/exon boundaries and the sequence is shown in SEQ. ID. No.: 1. All sequences are deposited with GenBank (accession Nos. M90556, M90557, M90558, M90559, M90560, M90561, and M90562).

Assembly and Characterization of the Gene Construct.

The beta casein hATIII transgene was constructed to resemble as accurately as possible the intact goat beta casein gene. The, vector was 14.95 Kb in length with 4.2 Kb of 5' flanking sequence and 5.3 Kb of 3' flanking sequence. The vector was engineered with three introns between exons 1–2, 7–8, and 8–9 to allow for splicing of the transcript. The introns were added to increase expression of the transgene (Brinster et al., Proc. Natl. Acad. Sci. USA., Devel. Biol. 85, pp.836–840, 1988.) and the 3' flanking sequence to decrease chromosomal position effects.

To allow for the cloning of various inserts, the beta casein promoter and 3' flanking region were reassembled from the subclones with unique restriction sites in exon 2 and exon 7. To remove the initiator methionine of the goat beta casein, the plasmid Bc106 was digested with the restriction enzyme TaqI. The TaqI site restricts the plasmid Bc106 six nucleotides upstream of the initiator methionine (TaqI site marked with ****, FIG. **10**). Following digestion with TaqI, the ends were filled in with Klenow DNA polymerase and a BamHI linker ligated into place. The ligation mixture was extracted with phenol-chloroform, chloroform-isoamyl alcohol, and ethanol precipitated as described in Maniatis, et al., 1989. The DNA was resuspended in digestion buffer and digested with EcoRI and BamHI. The resulting 1.5 Kb fragment was gel purified and ligated into pUC to form the plasmid Bc150. The entire available 5' region of the beta casein gene was constructed by sequential addition of fragments contained in the subclones Bc104, Bc147, and Bc103. The orientation of the Bc147 clone was verified by restriction mapping. The replacement of the 2.6 Kb XbaI fragment with the XbaI fragment from Bc147 was needed to replace a 31 bp deletion in the promoter between adjacent EcoRI restriction sites. To allow for the addition of the human antithrombin III cDNA, the BamHI site on the 3' end of Bc113 was converted to an XhoI site to form Bc114.

The 3' end of the goat beta casein gene was constructed in a similar fashion to the 5' end. The 1.8 Kb Bc107 clone was restricted with PpuMI to allow for the addition of a BamHI linker. The BamHI site was placed in exon 7 to include the intron between exons 7 and 8. The BamHI site is seven amino acids 5' to the termination of the goat beta casein mRNA but will have no effect on the hATIII since the cDNA for ATIII contains a termination signal. The BamHI/HindIII fragment spanning exon 7 from Bc107 was ligated into the vector Bc109 which contained the extreme 3' HindIII/SalI fragment to form Bc165. The 3' end was completed by the addition of the 4.4 Kb HindIII fragment from Bc108 to form the vector Bc118. The orientation of HindIII fragment from Bc108 was verified by restriction mapping. To allow for the addition of the hATIII cDNA, the 5' BamHI site in the vector Bc118 was changed to XhoI and the 3' SalI was converted to NotI to form Bc122.

In order to clone the entire hATIII cDNA into the goat beta casein expression vector, the 5' EcoRI restriction site was converted to an XhoI site using site-directed mutagenesis. The new vector designated pAT7 was sequenced to confirm the addition of the SalI site and the absence of any nucleotide changes in the 5' end. The hATIII cDNA was isolated as a 1.45 Kb XhoI/SalI fragment for cloning into the beta casein expression vector.

The 1.45KB XhoI/SalI hATIII cDNA was first ligated into XhoI digested Bc114 and Bc122 to form Bc143 and Bc144, respectively. The orientation of the cDNA was confirmed by sequencing and restriction analysis. The unique SacII restriction in the hATIII cDNA allows for the directional cloning of the intact goat beta casein hATIII vector. The complete transgene was assembled by ligating the SalI/SacI fragment from Bc143 into a SalI/SacII digested Bc144. The 5' and 3' junction of the hATIII cDNA were confirmed by sequencing to ensure no mutations had occurred. The final vector (Bc6) was 14.95 Kb in length and contained 4.2 Kb of 5' flanking region, exon 1, intron 1, part of exon 2, the 1.45 Kb human AT III cDNA, part of exon 7, intron 7, exon 8, intron 8, exon 9, and 5.3 Kb of 3' flanking sequence. The transgene was excised from the pUC backbone by digestion with SalI/NotI. The Bc6 transgene directs mammary specific expression of an approximately, 1.5 Kb hATIII-goat; beta casein hybrid mRNA containing the 5' and 3' untranslated regions of the goat beta casein mRNA and coding region of the hATIII mRNA.

Testing and Characterization of Gene constructs

Transgene constructs are generally tested in a mouse model system to assess their ability to direct high levels of expression and their ability to express in a tissue-specific manner.

The Bc6 transgene was prepared for microinjection by digestion of the Bc6 plasmid with SalI/NotI, removal of the bacterial sequences by TAE agarose gel electrophoresis followed by banding of the transgene on a cesium chloride gradient according to Lacy, et al., A Laboratory Manual, 1986. Cold Springs Harbor Laboratory, NY. <The 14.95 Kb transgene was then microinjected into mouse embryos to test the ability of the goat beta casein promoter to direct high level expression of recombinant human Antithrombin III

(rhATIII) into the milk. Seventeen transgenic mouse lines were generated as determined by Southern analysis. One mouse line showed a level of expression of rhAT III of 0.7–1.0 mg/ml as determined by Western blot analysis and a thrombin inhibition assay. Based on expression results and data collected from other ongoing mouse and goat experiments, the Bc6 transgene was microinjected into goat embryos for production of transgenic goats.

Generation and Characterization of Transgenic Animals

A founder (F_0) transgenic goat is defined as a viable transgenic animal resulting from embryo transfer of fertilized goat eggs that have been microinjected with a specified construct (8c6). Four founder Bc6 goats were produced. The general methodologies that follow in this section were used to generate all transgenic goats.

Goat Species and Breeds:

The transgenic goats produced for ATIII production are of Swiss origin, and are the Alpine, Saanen, and Toggenburg breeds.

Goat Superovulation:

The timing of estrus in the donors was synchronized on Day 0 by 6 mg subcutaneous norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, Kans.). Prostaglandin was administered after the first seven to nine days to shut down the endogenous synthesis of progesterone. Starting on Day 13 after insertion of the implant, a total of 18 mg of follicle-stimulating hormone (FSH-Schering Corp., Kenilworth, N.J.) was given intramuscularly over three days in twice-daily injections. The implant was removed on Day 14. Twenty-four hours following implant removal the donor animals were mated several times to fertile males over a two-day period (Selgrath, et al., Theriogenology, 1990. pp. 1195–1205).

Embryo Collection:

Surgery for embryo collection occurred on the second day following breeding (or 72 hours following implant removal). Superovulated does were removed from food and water 36 hours prior to surgery. Does were administered 0.8 mg/kg Diazepam (Valium®), IV, followed immediately by 5.0 mg/kg Ketamine (Keteset), IV. Halothane (2.5%) was administered during surgery in 2 L/min oxygen via an endotracheal tube. The reproductive tract was exteriorized through a midline laparotomy incision. Corpora lutea, unruptured follicles greater than 6 mm in diameter, and ovarian cysts were counted to evaluate superovulation results and to predict the number of embryos that should be collected by oviductal flushing. A cannula was placed in the ostium of the oviduct and held in place with a single temporary ligature of 3.0 Prolene. A 20 gauge needle was placed in the uterus approximately 0.5 cm from the uterotubal junction. Ten to twenty ml of sterile phosphate buffered saline (PBS) was flushed through the cannulated oviduct and collected in a Petri dish. This procedure was repeated on the opposite side and then the reproductive tract was replaced in the abdomen. Before closure, 10–20 ml of a sterile saline glycerol solution was poured into the abdominal cavity to prevent adhesions. The linea alba was closed with simple interrupted sutures of 2.0 Polydioxanone or Supramid and the skin closed with sterile wound clips.

Fertilized goat eggs were collected from the PBS oviductal flushings on a stereomicroscope, and were then washed in Ham's F12 medium (Sigma, St. Louis, Mo.) containing 10% fetal bovine serum (FBS) purchased from Sigma. In cases where the pronuclei were visible, the embryos were immediately microinjected. If pronuclei were not visible, the embryos were placed in Ham's F12 containing 10% FBS for short term culture at 37° C. in a humidified gas chamber containing 5% CO_2 in air until the pronuclei became visible (Selgrath, et al.,Theriogenology, 1990. p. 1195–1205).

Microinjection Procedure

One-cell goat embryos were placed in a microdrop of medium under oil on a glass depression slide. Fertilized eggs having two visible pronuclei were immobilized on a flame-polished holding micropipet on a Zeiss upright microscope with a fixed stage using Normarski optics. A pronucleus was microinjected with the Bc6 DNA construct in injection buffer (Tris-EDTA) using a fine glass microneedle (Selgrath, et al., Theriogenology, 1990. p. 1195–1205).

Embryo Development:

After microinjection, the surviving embryos were placed in a culture of Ham's F12 containing 10% FBS and then incubated in a humidified gas chamber containing 5% CO_2 in air at 37° C. until the recipient animals were prepared for embryo transfer (Selgrath, et al., Theriogenology, 1990. p. 1195–1205).

Preparation of Recipients:

Estrus synchronization in recipient animals was induced by 6 mg norgestomet ear implants (Syncromate-B). On Day 13 after insertion of the implant, the animals were given a single non-superovulatory injection (400 I.U.) of pregnant mares serum gonadotropin (PMSG) obtained from Sigma. Recipient females were mated to vasectomized males to ensure estrus synchrony (Selgrath, et al., Theriogenology, 1990. p. 1195–1205).

Embryo Transfer:

All embryos from one donor female were kept together and transferred to a single recipient when possible. The surgical procedure was identical to that outlined for embryo collection outlined above, except that the oviduct was not cannulated, and the embryos were transferred in a minimal volume of Ham's F12 containing 10% FBS into the oviductal lumen via the fimbria using a glass micropipet. Animals having more than six to eight ovulation points on the ovary were deemed unsuitable as recipients. Incision closure and post-operative care were the same as for donor animals (Selgrath, et al., Theriogenology, 1990. p. 1195–1205).

Monitoring of Pregnancy and Parturition:

Pregnancy was determined by ultrasonography 45 days after the first day of standing estrus. At Day 110 a second ultrasound exam was conducted to confirm pregnancy and assess fetal stress. At Day 130 the pregnant recipient doe was vaccinated with tetanus toxoid and Clostridium C&D. Selenium and vitamin E (Bo-Se) were given IM and Ivermectin was given SC. The does were moved to a clean stall on Day 145 and allowed to acclimatize to this environment prior to inducing labor on about Day 147. Parturition was induced at Day 147 with 40 mg of PGF_2a (Lutalyse®) purchased from Upjohn Company, Kalamazoo Mic. This injection was given IM in two doses, one 20 mg dose followed by a 20 mg dose four hours later. The doe was under periodic observation during the day and evening following the first injection of Lutalyse® on Day 147. Observations were increased to every 30 minutes beginning on the morning of the second day. Parturition occurred between 30 and 40 hours after the first injection. Following delivery the doe was milked to collect the colostrum and passage of the placenta was confirmed.

Verification of the Transgenic Nature of F_0 Animals:

To screen for transgenic F_0 animals, genomic DNA was isolated from two different cell lines to avoid missing any mosaic transgenics. A mosaic animal is defined as any goat that does not have at least one copy of the transgene in every cell. Therefore, an ear tissue sample (mesoderm) and blood sample were taken from a two day old F_0 animal for the

isolation of genomic DNA (Lacy, et al., A Laboratory Manual, 1986, Cold Springs Harbor, NY; and Herrmann and Frischauf, Methods Enzymology, 1987. 152: pp. 180–183). The DNA samples were analyzed by the polymerase chain reaction (Gould, et al., Proc. Natl. Acad. Sci, 1989. 86:p. 1934–1938) using primers specific for hATIII and by Southern blot analysis (Thomas, Proc Nati. Acad. Sci., 1980. 77:5201–5205) using a random primed hATIII cDNA probe (Feinberg and Vogelstein, Anal. Bioc., 1983. 132: pp. 6–13). Assay sensitivity was estimated to be the detection of one copy of the transgene in 10% of the somatic cells.

Generation and Selection of Production Herd

The procedures described above were utilized for production of the transgenic founder (F_0) goats, as well as other transgenic goats in our herd. The transgenic F_0 founder goats, for example, were bred to produce milk, if female, or to produce a transgenic female offspring if it was a male founder.

This transgenic founder male, was bred to non-transgenic females, and produced transgenic female offspring.

Transmission of Transgene and Pertinent Characteristics

Transmission of the Bc6 transgene in our goat line was analyzed in ear tissue and blood by PCR and Southern blot analysis. For example, Southern blot analysis of the founder male and the three transgenic offspring showed no rearrangement or change in the copy number between generations. The Southern blots were probed with the 1.45 Kb ATIII cDNA and a 0.38 Kb probe corresponding to goat beta casein exon 7. The blots were analyzed on a Betascope 603 and copy number determined by comparison of the transgene to the goat beta casein endogenous gene.

Evaluation of Expression Levels

The expression level of rhATIII in the milk of transgenic animals is determined using a thrombin inhibition assay, which measures the inhibition of thrombin's ability to remove a small peptide from an artificial substrate (S2238, Kabi, Franklin Ohio). The basis for this assay is described as follows. The interaction between ATIII and thrombin amounts to rapid irreversible inhibition of the protease by ATIII in the presence of heparin. However, the interaction is very slow in the absence of heparin. Attempts to extend the range of ATIII detectable on a single standard curve reveal that ATIII can only be determined accurately in stoichiometric titration across the linear range of standard curves. At low total thrombin concentration (0.7×10^{-9}M), the effective measuring range for ATIII is $0.15–0.75\times10^{-9}$M (~7.3–36.8 ng/ml). At high total thrombin, the effective measuring range for AT III is 0.25 to 1.25×10^{-9}M (~12–60 ng/ml) if the data are fit with a first degree polynomial, and 0.25 to 2.5×10^{-9}M (~12–120 ng/mi) if the data are fit with a second degree polynomial.

AT III activity assay

Materials:

1. 12x75 mm Glass Tubes;
2. Behring hATIII dilute to 0.5 mg/ml with diH_2O ($E^{0.1\%}280$=0.72);
3. Thrombin (100 units/vial diluted to 1.82 m is with 0.1 mg/ml BSA), purchased from Calbiochem, San Diego Calif.). Final Concentration 20 μg/ml;
4. Heparin (Sigma) from porcine intestinal mucosa 50 mg dissolved 5.0 mls diH_2O. Final Concentration 10 mg/ml;
5. Substrate (Kabi) S2238 25 mg diluted to 10 mls with diH_2O;
6. Dilution Buffer: 50 mM Tris, 150 mM NaCl, 0.1 mg/ml BSA pH 8.1;
7. Glacial Acetic Acid;
8. Repeat Pipettor; and
9. 37° C. circulating H_2O bath.

ASSAY SET-UP

Dilution of Stocks:

1. AT III stock to 1 μg/ml: 20μl ATIII+980 μls dilution buffer then 100 μl+900 μl dilution buffer;
2. Heparin/Buffer: 12.5 μl Heparin+25 mls dilution buffer; and
3. Thrombin stock: 1:10 dilution into dilution buffer (2.0 mls for a 30 tube assay).

Standard Curve Set-Up

Final Concentration AT III	Diluted AT III Stock	Dil Buffer	Heparin Buffer	Thrombin
0 ng/ml	0 μl	100 μl	750 μl	50 μl
5 ng/ml	5 μl	95 μl	750 μl	50 μl
10 ng/ml	10 μl	90 μl	750 μl	50 μl
20 ng/ml	20 μl	80 μl	750 μl	50 μl
30 ng/ml	30 μl	70 μl	750 μl	50 μl
40 ng/ml	40 μl	60 μl	750 μl	50 μl
50 ng/ml	50 μl	50 μl	750 μl	50 μl
60 ng/ml	60 μl	40 μl	750 μl	50 μl

1. Incubate the above at 37° C. for 15 minutes;
2. Then add 100 μl of substrate to each tube using a repeat pipettor and incubate for 15 minutes at 3720 C.;
3. Stop with 50 μl of glacial acetic acid to each tube; and
4. Read at 405 nm. Blank with 150 μl Buffer, 750 μl Heparin Buffer, 100 μl substrate and 50 μl Acetic acid stopped at time zero (NOT INCUBATED).

Calculations: Use a second order polynomial curve fit for the standard curve, plug A 405 nm readings into the equation. Divide by the volume of sample used in the assay tube in mls and multiply by the appropriate inverse dilution factor for the sample.

Characterization of Human ATIII Produced in the Milk of a Transgenic Goat.

TgATIII was purified from goat milk by adaptation of methods previously developed for extracting ATIII from mouse milk and human blood. Other more novel methods for separating proteins from milk are also in development. The method described is the earliest method that we utilized. Milk from one BC6 goat contained tgATIII at a concentration estimated to be 0.08 mg/ml by ELISA. Other goats with much higher expression levels (4 to 6 mg/ml) were also developed and milk from those goats was purified using similar methods. Small scale purification was performed by the following method.

ATII Purification Process

ATIII Extraction/Clarification

Thaw Milk

Add an equal volume of 2M Arginine

Centrifuge at 8000 rpm for 30 minutes and remove the fat pad on the top

Filter through a 0.2 μm nominal polypropylene filter

Dilute the conductivity to less than 20 mS/cm (approximately a 1/7 dilution)

Expected step yield of AT III is ≧295%

Heparin-Hyper D™ Chromatography

Equilibrate with 3 column volumes of 20 mM sodium phosphate, 50 mM NaCl, pH7.0

Load, the target operational capacity is approximately 8 mg of ATIII/mL of resin

Wash with 4 column volumes of Equilibration Buffer

Step Elute with 4 column volumes of 20 mM sodium phosphate, 3.0 M NaCl, pH7.0

Dilute with 9 volumes of 1.5 M sodium citrate

Expected step yield of AT III is ≧95%

Ether-PoroS™ 50 Chromatography

Equilibrate with 3 column volumes of 1.40M sodium citrate, pH7.0

Load, the target operational capacity is approximately 4 mg of ATIII/mL of resin

Wash with 4 column volumes of Equilibration Buffer

Step Elute with 4 column volumes of 1.10M sodium citrate, pH7.0

Expected step yield of AT III is ≧275%

Ultrafiltration with Amicon YM10 Spiral Crossflow

Concentrate to approximately 20 mg/mL (160 units/mL) and buffer exchange into 10 mM

ammonium bicarbonate, pH 7.4.

Expected step yield of AT III is ≧95%

Lyophilize

Reconstitute into buffer of choice

Expected step yield of ATIII is ≧295%

A sample of the concentrated pool was examined by SDS-PAGE (10% separating gel) with a human ATIII standard for comparison and estimation of purity. The purity is estimated to be >98% (see FIG. 2.). The UV/Vis spectrum of tgATIII is indistinguishable (within experimental error) from that of plasma derived hATIII. N-terminal sequence analysis gave the predicted sequence for hATIII. Reversed phase HPLC analysis showed threepeaks compared to two peaks for plasma ATIII (FIG. 1). All three peaks were found to be ATIII by N-terminal sequence analysis. Peak #1 is miniscule in the tgATIII and not apparent in the plasma derived ATIII. Peak 2 is present in both samples and varies with preparations. Peak three is the predominant peak. Examination of specific peaks from FIG. 1 following digestion with lysylendopeptidase and separation by reverse phase HPLC to create peptide maps identified the presence of oxidized methionine residues in the two early small peaks of the tgATIII. The middle peak contained oxidized methionines mainly on a single peptide (K30 on table 2), whereas the earliest peak contained oxidized methionines on most of the peptides that contained methionine. The major peak did not contain any oxidized methionines as measured by the technique being used above. The presence of oxidized methionines on this ATIII may be a function of the processing of the molecule following removal of the milk from the goat.

Monosaccharide compositional analysis indicating the amounts of fucose (Fuc), GalNac, N-Acetylglucosamine (GlcNAc), Galactose (Gal), Mannose (Man) and Sialic Acid is shown below in Table 1.

TABLE 1

	mole sugar/mole protein					
Sample	Fuc	GalNAc	GlcNAc	Gal	Man	Sialic Acid
phATIII	0.39	0.00	20.70	11.74	14.90	11.80
tATIII	3.18	2.39	14.75	6.44	18.18	5.21

Lysylendopeptidase maps of plasma derived hATIII and tgATIII have been analyzed by liquid chromatography/mass spectrometry (LC/MS) to compare the amino acid backbone structure, the glycosylation profile and to confirm the identity of the disulfide linked peptides. For non-reduced human plasma ATIII and tgATIII, 32 of the 35 predicted peptides were identified, including the three pairs of disulfide linked peptides. The disulfide cross-linking was identical in the plasma ATIII and the tgATIII as shown in the peptide maps of the non-reduced proteins (FIG. 3). The peaks labeled with the arrows are the peaks containing disulfide cross-linked peptides. These peaks have identical retention times for each disulfide crosslinked peptide pair of each protein. The remaining peaks on the maps are identical with the exception of the glycosylated peptides. The maps of the reduced, pyridylethylated ATIII digests detail this variability more specifically.

For reduced and pyridylethylated plasma and transgenic hATIII, 33 of the 35 predicted peptides were identified by LC/MS. Chromatograms of the plasma ATIII and the individual forms of the tgATIII can be seen in FIG. 4 with the identification of the individual equivalent peptides by mass spectrometry shown in Table 2 below. Again, all of the peptides are identical with the exception of the glycosylated peptides.

TABLE 2

LC/MS ANALYSIS of ATIII PEPTIDE MAPS

PEPTIDE	RESIDUES	THEORETICAL MASS	phATIII	tgATIII
K1	1–11	1232.4	1232.0	1232.3
K2	12–28	2152.6	2152.6	2152.3
K3	29–29	146.2	ID	ID
K4	30–39	1093.1	1092.9	1092.8
KS	40–53	1699.0	1698.7	1698.7
K6	54–70	1957.1	1956.9	1956.9
K7	71–91	2299.6	2299.3	2299.4
K8	92–107	GP	GP	GP
K9	108–114	838.9	838.7	838.7
K20	115–125	1340.5	1340.3	1340.2
K11	126–133	1170.5	1170.1	1170.1
K12	134–136	GP	GP	GP
K13	137–139	320.4	ND	ND
K14	140–150	1219.4	1219.1	1219.4
K15	151–169	GP	GP	GP
K16	170–176	860.0	860.2	860.0
K17	177–188	1330.4	1330.4	1330.2
K18	189–193	GP	GP	GP
K19	194–222	3248.8	3248.8	3248.8
K20	223–226	502.6	502.9	502.8
K21	227–228	233.3	ND	ND
K22	229–236	978.1	978.1	977.9
K23	237–241	698.8	698.5	698.6
K24	242–257	1799.0	1798.8	1798.9
K25	258–275	2209.6	2209.3	2209.6
K26	276–287	1314.6	ID	ID
K27	288–290	372.4	ID	ID
K28	291–294	417.5	417.3	417.5
K29	295–297	374.5	374.2	374.2
K30	298–332	4261.0	4261.1	4260.9
K31	333–348	1849.1	1849.0	1848.9
K32	349–350	233.3	ID	ID
K33	351–370	2202.5	2202.5	2202.2
K34	371–403	3448.9	3448.7	3448.7
K35	404–432	3421.2	3421.2	3421.2
K3–4	29–39	1221.3	1221.4	1221.1
K26–27	276–290	1669.0	1668.8	1668.9
K32–33	349–370	2417.7	2417.5	2417.6

GP = Glycopeptide
ND = Not detected (m/z 2+ below scan range)
ID = Incomplete digestion product
phATIII = Human Plasma ATIII
tgATIII = Transgenic (goat) Human ATIII

The glycopeptide peak retention times (glycopeptide peaks are labeled with K#'s on FIG. 4) did not vary significantly from those of the plasma derived protein, but the chromatograms show that peak widening and peak splitting did occur due to the variability in the oligosaccharide residues attached to the glycopeptides.

Based on the LC/MS, peptide mapping, and carbohydrate composition data, the only differences observed between the tgATIII and the plasma derived hATIII arise due to the different patterns of ggycosylation. Despite the presence of N-Acetylgalactosamine (GalNAc) in the monosaccharide analysis, no O-linked glycosylation was present on the transgenic protein, suggesting the presence of oligosaccharides different than found on the plasma derived human ATIII.

The major glycoform at each glycosylation site of the plasma derived (pATIII) is a complex type oligosaccharide and has a mass corresponding to HexNAc4, Hex5, NANA2 which is in agreement with the reported structure GlcNAc4, Man3, Gal2, NANA2. With the exception of Asn_{155}, located on K15, the major glycoforms on tgATIII contain oligosaccharides of the complex type. The major glycoform at each of the complex oligosaccharide containing sites on tgATIII has a mass corresponding to HexNAc4, Hex6, NANA1 which is in agreement with the structure GlcNAc4, Fucl, Man3, Gal2, NANA1 with the second most abundant form being the disialylated form of the same structure, HexNAc4, Hex6, NANA2 which is in agreement with the structure GlcNAc4, Fucl, Man3, Gal2, NANA2

TgATIII contains 'a significant amount of oligomannose type and hybrid forms at Asn_{155} and only a very low level of hybrid structures at the other locations. Oligomannose type structures are more primitive structures that are remodeled into the complex type oligosaccharides in the endoplasmic reticulum. Oligomannose structures display masses ranging from Hex5 to HexNAc2, Hex9. These values agree with structures comprised of 5 up to GlcNAc2, Man9, with only the number of mannose residues varying. Hybrid oligosaccharides contain elements of complex oligosaccharides on one antenna of an individual glycosylation site and components of oligomannose type oligosaccharides on the other antenna. The N-linked glycosylation for tgATIII was much more heterogeos than phATIII, with a higher degree of fucosyation and more varied sialylation (Table 3). Several glycoforms with a mass difference of 41 were observed by LC/MS which can be accounted for by the substitution of a Hexose residue by a HexNAc (Tables 4). In view of the monosaccharide composition (Table 1) and the lack of O-linked glycosylation (based on comparison of the observed vs theoretical mass for all peptides other than those containing an N-linked site) this could be accounted for by the substitution of one or more galactose residues by GalNAc . This result was confirmed by fragmentation analysis mass spectroscopy of isolated glycopeptides. FIG. 5 is an example of the mass spectrometry data for one of the individual glycopeptide residues from phATIII. It's pattern is fairly simple. In contrast, FIG. 6 is an example of the same glycopeptide from tgATIII. The increased complexity of the pattern is obvious, and can be deciphered into specific structures as described above.

Several glycoforms with mass differences of 16 mass units were also identified. The difference is explained by the presence of an oxidized form of sialic acid, N-Glycolylneuraminic acid (NGNA) in place of N-Acetylneuraminic acid (NANA). NGNA is a commonform of sialic acid found in goats. Approximately 25% of the sialic acids found in tgATIII are NGNA. Approxniately 25% of goat plasma ATIII slalic acid is NGNA (FIG. 7).

Thus, we have determined that; (1) one of the four glycosylation sites on tgATIII has mainly high mannose (oligomannose) and hybrid type oligosaccharide structures, whereas the phATIII has biantenarry, complex oligosaccharides on each of the four sites; (2); the complex oligosaccharides of tgATIII are not fully sialylated, whereas the phATIII oligosaccharides are fully sialylated; (3) the tgATIII has a percentage of its sialic acid that is NGNA whereas the phATIII has only NANA; and ((4) tgATIII contains N-acetylgalactosamine on its N-linked oligosaccharides and the phATIII does not; and (5) the tgATIII has fucose on its proximal GlcNAc on each of the three sites having complex oligosaccharides, whereas the phATIII has only a very small amount of fucose on any site.

The tgATIII exhibits a faster clearance time in rabbits, mice and monkeys than does phATIII. Twenty ug samples of test ATIII was injected via the tail vein and residual ATIII determined using an ELISA assay which has little cross reactivity with mouse ATIII. The pattern shown in FIG. 8 for clearance in mice mimics the pattern found for the same materials in rabbits. The clearance appears to be bimodal and is approximately 10 times faster than for hATIII. In vivo clearance was also examined in a monkey model system. Both trace and high levels of radioiodinated ATIII were injected and detected in plasma samples by counting in a gamma counter. The clearance pattern of tgATIII in monkeys indicated only a 4 to 5-fold faster clearance (FIG. 9) from the circulation than the phATIII and could also be defined by a biphasic mechanism.

Early experiments indicate that the tgATIII may have a stronger affinity for heparin than the phATIII. This would be important since ATIII inhibits thrombin at inflamation or injury sites binding to heparan sulfate in the endothelial layer to the vasculature. Once bound its affinity for thrombin is enhanced 1000 fold and it binds to and irreversibly inhibits thrombin.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3515 base pairs
(B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGAATTTACC CCAAGATCTC AAAGACCCAC TGAATACTAA AGAGACCTCA TTGTGGTTAC 60

AATAATTTGG GGACTGGGCC AAAACTTCCG TGCATCCCAG CCAAGATCTG TAGCTACTGG 120

ACAATTTCAT TTCCTTTATC AGATTGTGAG TTATTCCTGT TAAAATGCTC CCCAGAATTT 180

CTGGGGACAG AAAAATAGGA AGAATTCATT TCCTAATCAT GCAGATTTCT AGGAATTCAA 240

ATCCACTGTT GGTTTTATTT CAAACCACAA AATTAGCATG CCATTAAATA CTATATATAA 300

ACAGCCACTA AATCAGATCA TTATCCATTC AGCTTCTCCT TCACTTCTTC TCCTCTACTT 360

TGGAAAAAAG GTAAGAATCT CAGATATAAT TTCAGTGTAT CTGCTACTCA TCTTTATTTT 420

GGACTAGGTT AAAATGTAGA AAGAACATAA TTGCTTAAAA TAGATCTTAA AAATAAGGGT 480

GTTTAAGATA AGGTTTACAC TATTTTCAGC AGATATGTTA AAAAATAGAA GTGACTATAA 540

AGACTTGATA AAAATTATAG GTGACTGCAA TTTTTGCCAT GAGGTTTGCA GGATCTTGGT 600

TCCCTGACCA GGGATCAAAC CTGCACTCCC CTGGAAGCAT GGAGTCTTGG ACATTTGTAT 660

TATACACTAT CTTTGGTTCC TTTTAAAGGG AAGTAATTTT ACTTAAATAA GAAAATAGAT 720

TGACAAGTAA TACGCTGTTT CCTCATCTTC CCATTCACAG GAATCGAGAG CCATGAAGGT 780

CCTCATCCTT GCCTGTCTGG TGGCTCTGGC CATTGCAAGA ACAAAGAGGT AAATACAGAA 840

AAAATGTTGA AATAATAGAC TAGTACTGTC TGCCTATGTG TAGAAATCAC ATTACCAACA 900

TCATAAATGT ATAAATAATG CACAATCTCA GATTTATTTT TTAATGCTAA GAAAGTCATT 960

TATGTTCATC CACTATCTCA ACAGTATCCT ATAGGACCAC AACTCTGGGT CAAGTGCTTT 1020

CTATAGTATT GTACCATCTG TACCATCAAT TCCTAAAGAA AAAGGAAAAG AAACCAATAA 1080

GCAACAGACC AACAAGAAGG AACACAGACA AGAACAAAAA ATGAGTAATA TTGTACAAAT 1140

ACAATTGCAC GCTGCAGGAA CTAAAGTGTT TTTTTTTCTC TCTCTTTTTA GCAGGAAGAA 1200

CTCAATGTAG TCGGTGAGGT AAGATGATTT TTATACAAAG AAAAAAATTA ATTTAACTGT 1260

AAAATAGTAA CAGACTCTGA TGATCTAGCA GAAAACTCAG CTAATTGTCA ATTTTTATTT 1320

TTCCTTTATA GACTGTGGAA AGCCTTTTCA AGCAGTGAGG TAAGATAATG TTCATTCAGA 1380

GGCAATTTCC CAGATTTAGA GCAATAAAAC ACTGTATTAT CTTTTTGTGT TACATTAATT 1440

GGCAACCCAC TCCAGTACTC TTGACAAATA TGAATTTTTC TTTAAAGCTA AACCTGATTT 1500

TATTTTTATT TTTCCAAAGG AATCTATTAC ACACATCAAT AAGGTAAAAC CTTCATATTT 1560

AAACGTATCA TTTTTAAAAA TTTCATGTTT GATTTTTATA AACAGCATTT CTTTATGTGT 1620

GATTTTTTTT TTTACCAGAA AATTGAGAAG TTTCAAAGTG AGGAACAACA GCAAACAGAG 1680

GTAATTTGTT TCACTATGAG TATATTTTGA GAAGTATTAT GAAACATAAC ACATAAAAGA 1740

TTTATAATAA TTATGTTCAG TCTAAGAATG GTAATATAAG TGTCAGTGTA AGAAATGAAA 1800

ACTTTGACAA AATGAAAATA TTTTAAAGAT AGAAACACAT TTTTAAACAC ATAATCAAAT 1860

TTCAGAGTAT AGAATAAATA CCCAAGAATA ACTACTGGTA TATTCATTTT ACTAATGGTA 1920

TACCTGGCTT TAATAAATGC ATATTAGTAG GAACAATTCC AGACTAGGGA CTGTGATCCC 1980

-continued

```
CTTATTCTAA TGATGGATAT GCTGATGAAA GACAGTAGGG TGACAGTGTG GCACTAATCC   2040

TAATAAATGG AAGATTTTCT TTCTCTCTCT TCACTGAATT ATGTTTTAAA AAGAGGAGGA   2100

TAATTCATCA TGAATAACAA TTATAACTGG ATTATGGACT GCAAAGGCAT TGGTTTTCCT   2160

TCTTTCCAGG ATGAACTCCA GGATAAAATC CACCCCTTTG CCCAGGCACA AAGTCTCTAG   2220

TCTATCCCTT CACTGGGCCC ATCCCTAACA GCCTCCCACA AAACATCCTG CTGCTCTTAC   2280

TCAAACCCCT GTGGTGGTGC CGCCTTTCCT TCAGCCTGAA ATAATGGGAG TCCTTGCCAA   2340

AGTGAAGGAG ACTATGGTTC CTAAGCACAA AGAAATGCCC TTCCCTAAAT ATCCAGTTTG   2400

AGCCCTTTAC TGAAAGCCAG AGCCTGACTC TCACTGATGT TGAAAAGCTG CACCTTCTTT   2460

CTCTGCCTCT GGTCCAGTCT TGGATGCACC AGCCTCCCCA GCCTCTTTCT CCAACCGTCA   2520

TTGTTTCCTC CTCAGTCCGT GCTGTCCCTT TCTCAGCCCA AAGTTCTGCC TGTTCCCCAG   2580

AAAGCAGTGC CCCAGAGAGA TATGCCCATC CAGGCCTTTC TGCTGTACCA GGAGCCTGTA   2640

CAATTGGTCC TGTCCGGGGA CCCTTCCCTA TTCTTGTAAG TCTAAATTTA CTAACTGTGC   2700

TGTGGTTAAC TTCTGATGTT TGTATGATAT TTGAGTAATT AAGAGCCCTA CAAAAAAATC   2760

AATAATGAAT GGTTCCAAAA TAAGCATAGC TGAGATTAAT GATTCTCAGC ATTAGTTATA   2820

AATAGAATAA GCTGGAAAAC CTTCACCTCC CCTCCACCAC CAGATCCCAA AAACAAAATA   2880

CTGAAGATGC TTATTTCAAT ACTCAGGGAA AATTTTCTTG CCAAAAAGGC AAGAATTGTA   2940

TAATTCATTC ACTTATTTTA TTTTTTTTAA TTTTTAAGGT CTAAGAGGAT TTCAAAGTGA   3000

ATGCCCCCTC CTCACTTTTG GTCAAATTGG AAATGGGGGT GAGATGAAGA GTTATAACAT   3060

ATAACTAAAT GGACATTGTT CTCTATTCCA CAGAATTGAC TGCGACTGGA AATATGGCAA   3120

CTTTTCAATC CTTGCATCAT GCTACTAAGA TAATTTTTAA ATGAGTATAC ATGGAACAAA   3180

AAATGAAACT TTATTCCTTT ATTTATTTTA TGCTTTTTCA TCTTAATTTG AATTTGAGTC   3240

ATAAACCATA TACTTTCAAA ATGTTAATTC AACATTAGCA TAAAAGTTCA ATTTTAACTT   3300

GGAAATATCA TGAACATATC AAATTATGTA TAAAAATAAT TTCTGGAATT GTGATTATTA   3360

TTTCTTTAAG AATCTATTTC CTAACCAGTC ATTTCAATAA ATTAACCCTT AGGCATATTT   3420

AAGTTTTCTT GTCTTTATTA TATTTTTAAA AATGAAATTG GTCTCTTTAT TGTTAACTTA   3480

AATTTATCTT TGATGTTAAA AATAGCTGTG GAAAA                              3515
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Val Leu Ile Leu Ala Cys Leu Val Ala Leu Ala Ile Ala Arg
  1               5                  10                  15

Glu Gln Glu Glu Leu Asn Val Val Gly Glu Thr Val Glu Ser Leu Ser
            20                  25                  30

Ser Ser Glu Glu Ser Ile Thr His Ile Asn Lys Lys Ile Glu Lys Phe
        35                  40                  45

Gln Ser Glu Glu Gln Gln Gln Thr Glu Asp Glu Leu Gln Asp Lys Ile
    50                  55                  60

His Pro Phe Ala Gln Ala Gln Ser Leu Val Tyr Pro Phe Thr Gly Pro
65                  70                  75                  80
```

-continued

```
Ile Pro Asn Ser Leu Pro Gln Asn Ile Leu Pro Leu Thr Gln Thr Pro
                85                  90                  95

Val Val Val Pro Pro Phe Leu Gln Pro Glu Ile Met Gly Val Pro Lys
            100                 105                 110

Val Lys Glu Thr Met Val Pro Lys His Lys Glu Met Pro Phe Pro Lys
        115                 120                 125

Tyr Pro Val Glu Pro Phe Thr Glu Ser Gln Ser Leu Thr Leu Thr Asp
    130                 135                 140

Val Glu Lys Leu His Leu Pro Leu Pro Leu Val Gln Ser Trp Met His
145                 150                 155                 160

Gln Pro Pro Gln Pro Leu Ser Pro Thr Val Met Phe Pro Pro Gln Ser
                165                 170                 175

Val Leu Ser Leu Ser Gln Pro Lys Val Leu Pro Val Pro Gln Lys Ala
            180                 185                 190

Val Pro Gln Arg Asp Met Pro Ile Gln Ala Phe Leu Leu Tyr Gln Glu
        195                 200                 205

Pro Val Leu Gly Pro Val Arg Gly Pro Phe Pro Ile Leu Val
    210                 215                 220
```

What is claimed is:

1. Mammary gland produced antithrombin III having a monosaccharide composition which comprises GalNAc.

2. Mammary gland produced antithrombin III having a monosaccharide composition which comprises fucose.

3. Mammary gland produced antithrombin III having a monosaccharide composition which comprises a higher level of mannose than plasma derived antithrombin III.

4. The mammary gland produced antithrombin III of claim 1 further comprising fucose.

5. The mammary gland produced antithrombin III of claims 1, 2, or 4 wherein the antithrombin III further comprises a higher level of mannose than plasma derived antithrombin III.

6. The mammary gland produced antithrombin III of claim 1, 2, 3, or 4 wherein the antithrombin III is produced in the mammary glands of a transiemc goat.

7. A method for producing antithrombin III in mammalian milk, comprising:

a. providing a transgenic mammal that expresses in its mammary tissue a transgene which encodes a human antithrombin III with a monosaccharide composition which comprises GalNAc, wherein said human antithrombin III is secreted into the milk of the mammal; and

b. collecting milk from the transgenic animal which contains the human antithrombin III;

c. to thereby obtain human antithrombin III with a monosaccharide composition which includes GalNAc.

8. The method of claim 7, further comprising isolating human ATIII from the milk.

9. The method of claim 7, wherein the transgenic mammal is goat.

10. The method of claim 7, wherein the transgenic mammal is a mouse.

11. A glycosylated human antithrombin III which is produced in mammary gland of a non-human transgenic mammal, wherein the antithrombin III comprises a monosaccharide composition which comprises GalNAc.

12. A glycosylated human antithrombin III which is produced in the mammary gland of a non-human transgenic mammal, wherein the antithrombin III comprises a monosaccharide composition which comprises fucose.

13. A glycosylated human antithrombin III which is produced in the mammary gland of a non-human transgenic mammal, wherein the antithrombin III comprises a monosaccharide composition which comprises a higher level of mannose than plasma derived antithrombin III.

14. The glycosylated human antithrombin III of claim 13 wherein the antithrombin III has a higher affinity for heparin binding as compared to plasma derived antithrombin III.

15. A method for producing antithrombin III in mammalian milk, comprising:

providing a transgenic mammal that expresses in its mammary tissue a transgene which encodes a human antithrombin III with a monosaccharide composition which comprises fucose, wherein said human antithrombin III is secreted into the milk of the mammal; and

collecting milk from the transgenic animal which contains the human antithrombin III

to thereby obtain human antithrombin III with a monosaccharide composition which includes fucose.

16. The method of claim 15, wherein the transgenic mammal is a goat.

17. The method of claim 15, wherein the transgenic mammal is a mouse.

18. A method for producing antithrombin III in mammalian milk, comprising:

providing a transgenic mammal that expresses in its mammary tissue a transgene which encodes a humanantithrombin III having a monosaccharide composition which comprises a higher level of mannose than plasma derived antithrombin III, wherein said human antithrombin III is secreted into the milk,of the mammal; and

collecting milk from the transgenic animal which contains the human antithrombin III to thereby obtain human antithrombin III having a monosaccharide composition which includes a higher level of mannose than plasma derived antithrombin III.

19. The method of claim 18, wherein the transgenic mammal is a goat.

20. The method of claim 18, wherein the transgenic mammal is a mouse.

* * * * *

Attorney's Docket No.: 10275-045002

B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : DiTullio et al.
Serial No. : 09/143,155
Filed : August 28, 1998
Title : TRANSGENICALLY PRODUCED ANTITHROMBIN III

Art Unit : 1632
Examiner : G. Lee

Commissioner for Patents
Washington, D.C. 20231

TERMINAL DISCLAIMER UNDER 37 CFR §§3.73(b) AND 1.321(b)

Pursuant to 37 CFR §3.73(b), Genzyme Transgenics Corporation, a corporation, certifies that it is the assignee of the entire right, title, and interest in the above application by virtue of:

An assignment from the inventors of the patent application identified above. The assignment was recorded in the Patent and Trademark Office at Reel 7592, Frame 0745 on May 25, 1995, or a copy thereof is attached.

The undersigned has reviewed all the documents in the chain of title of the above-identified application and to the best of undersigned's knowledge and belief, title is in the assignee identified above.

The undersigned (whose title is supplied below) is empowered to act on behalf of the assignee.

Pursuant to 37 CFR §1.321(b), and to obviate a double patenting rejection, the assignee identified above hereby waives and disclaims the terminal portion of the term of the entire patent to be granted upon the above identified application subsequent to the expiration date of U.S. Patent No. 5,843,705. Further, any patent granted on the above-identified application shall be enforceable only for and during such period that said patent is commonly owned with U.S. Patent No. 5,843,705.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

June 21, 2000
Date of Deposit

Anne M. Guerra
Signature

Anne M. Guerra
Typed or Printed Name of Person Signing Certificate

The assignee identified above does not disclaim any terminal part of any patent granted on the above identified application prior to the expiration date of the full statutory term of U.S. Patent No. 5,843,705 in the event that it later: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR §1.321, has all claims cancelled by a reexamination certificate, or is otherwise terminated prior to expiration of its statutory term, except for the separation of legal title as stated above.

This disclaimer runs with any patent granted on the above application and is binding upon the grantee, its successors or assigns.

Enclosed is a check for $110 for the required fee pursuant to 37 CFR §1.20(d).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12 June 2000

Sandra Nusinoff Lehrman

Sandra Nusinoff Lehrman
Title: President & CEO

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

C

Return To:
USPTO Home Page
Finance Online Shopping Page

United States Patent and Trademark Office

Maintenance Fee Statement

03/27/2009 11:34 AM EDT

Patent Number: 6441145

Customer Number: 23628

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON MA 02210-2206

According to the records of the U.S.Patent and Trademark Office (USPTO), the maintenance fee and any necessary surcharge have been timely paid for the patent listed below. The "PYMT DATE" column indicates the payment date (i.e., the date the payment was filed).

The payment shown below is subject to actual collection. If the payment is refused or charged back by a financial institution, the payment will be void and the maintenance fee and any necessary surcharge unpaid.

Direct any questions about this statement to: Mail Stop M Correspondence, Director of the USPTO, P.O.Box 1450, Alexandria, VA 22313-1450.

PATENT NUMBER	FEE AMT	SUR-CHARGE	PYMT DATE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	ATTY DKT NUMBER
6,441,145	$450.00	$0.00	02/27/06	09/143,155	08/27/02	08/28/98	04	YES	G0744.70047US01

Click here to obtain your Maintenance Fee Statement as a PDF.
Please note that in order to view your Maintenance Fee Statement as a PDF, you must have Adobe's Acrobat Reader installed. If you do not have it, download it for FREE!

Get Acrobat Reader

Need Help? | USPTO Home Page | Finance Online Shopping Page

D

CLAIM	PACKAGE INSERT/REFERENCE INFORMATION
1. Mammary gland produced antithrombin III	Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk. (Page 10*, Package Insert) The human AT cDNA was derived from a modified pBAT6 plasmid,[31] which allows excision of the cDNA as an *Xho* I to *Sal* I fragment. (Page 4561, first paragraph of the "Materials and Methods" section, *Blood*, Vol. 91, No. 12, 1998: 4561-4571, referencing Broker et al.: Expression of human antithrombin III in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Biochim Biophys Acta* 908:203, 1997)
having a monosaccharide composition which comprises GalNAc.	RhAT contained a GalNAc for galactose substitution on some N-linked oligosaccharides, as well as a high degree of fucosylation. (Abstract, *Blood*, Vol. 91, No. 12, 1998: 4561-4571)
7. A method for producing antithrombin III in mammalian milk, comprising:	Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk. (Page 10*, Package Insert) The human AT cDNA was derived from a modified pBAT6 plasmid,[31] which allows excision of the cDNA as an *Xho* I to *Sal* I fragment. (Page 4561, first paragraph of the "Materials and Methods" section, *Blood*, Vol. 91, No. 12, 1998: 4561-4571, referencing Broker et al.: Expression of human antithrombin III in Saccharomyces cerevisiae

* Page numbers were added by applicant to the Package Insert.

	and Schizosaccharomyces pombe. *Biochim Biophys Acta* 908:203, 1997)
a. providing a transgenic mammal that expresses in its mammary tissue a transgene which encodes a human antithrombin III	Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk. (Page 10*, Package Insert) The human AT cDNA was derived from a modified pBAT6 plasmid,[31] which allows excision of the cDNA as an *Xho* I to *Sal* I fragment. (Page 4561, first paragraph of the "Materials and Methods" section, *Blood*, Vol. 91, No. 12, 1998: 4561-4571, referencing Broker et al.: Expression of human antithrombin III in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Biochim Biophys Acta* 908:203, 1997)
with a monosaccharide composition which comprises GalNAc,	RhAT contained a GalNAc for galactose substitution on some N-linked oligosaccharides, as well as a high degree of fucosylation. (Abstract, *Blood*, Vol. 91, No. 12, 1998: 4561-4571)
wherein said human antithrombin III is secreted into the milk of the mammal;	Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk. (Page 10*, Package Insert)
and b. collecting milk from the transgenic animal which contains the human antithrombin III;	The milk from two of the (F_1) transgenic goats was pooled and used in this study. (Page 4561, col 2, *Blood*, Vol. 91, No. 12, 1998: 4561-4571)

* Page numbers were added by applicant to the Package Insert.

c. to thereby obtain human antithrombin III with a monosaccharide composition which includes GalNAc.	RhAT contained a GalNAc for galactose substitution on some N-linked oligosaccharides, as well as a high degree of fucosylation. (Abstract, *Blood*, Vol. 91, No. 12, 1998: 4561-4571)

* Page numbers were added by applicant to the Package Insert.

E

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use ATryn safely and effectively. See full prescribing information for ATryn.

ATryn, Antithrombin (Recombinant)
Lyophilized powder for reconstitution
Initial U.S. Approval: 2009

INDICATIONS AND USAGE

ATryn is a recombinant antithrombin indicated for the *prevention* of peri-operative and peri-partum thromboembolic events in hereditary antithrombin deficient patients. (1)

It is not indicated for *treatment* of thromboembolic events in hereditary antithrombin deficient patients.

DOSAGE AND ADMINISTRATION

- **For intravenous use only after reconstitution**
- The dosage of ATryn is individualized for each patient. Treatment goal is to restore and maintain functional antithrombin (AT) activity levels between 80% - 120% (0.8 - 1.2 IU/mL) of normal.
- Administer loading dose as a 15-minute intravenous infusion immediately followed by continuous infusion of the maintenance dose. (2.2)

	Loading Dose (IU)		Maintenance Dose (IU/hour)	
Surgical Patients	$\frac{(100 - \text{baseline AT activity})}{2.3}$	x Body Wt (kg)	$\frac{(100 - \text{baseline AT activity})}{10.2}$	x Body Wt (kg)
Pregnant Women	$\frac{(100 - \text{baseline AT activity})}{1.3}$	x Body Wt (kg)	$\frac{(100 - \text{baseline AT activity})}{5.4}$	x Body Wt (kg)

- AT activity monitoring is required for proper treatment. Check AT activity once or twice per day with dose adjustments made according to table below. (2.2)

Initial Monitor Time	AT Level	Dose Adjustment	Recheck AT Level
2 hours after initiation of treatment	< 80%	Increase 30%	2 hours after each dose adjustment
	80% to 120%	None	6 hours after initiation of treatment or dose adjustment
	> 120%	Decrease 30%	2 hours after each dose adjustment

- Continue administration of ATryn until adequate follow-on anticoagulation has been established.
- Store at 2-8°C (36-46°F). Discard any unused portion. (2.1, 16)

DOSAGE FORMS AND STRENGTHS

- ATryn is a sterile lyophilized powder for reconstitution. Each single dose vial of ATryn contains the potency as stated on the label, which is approximately 1750 IU. (3)

CONTRAINDICATIONS

- Known hypersensitivity to goat and goat milk proteins. (4)

WARNINGS AND PRECAUTIONS

- Anaphylaxis and severe hypersensitivity reactions are possible. Should symptoms occur, treatment with the product should be discontinued, and emergency treatment should be administered. (5.1)
- The anticoagulant effect of drugs that use antithrombin to exert their anticoagulation may be altered when ATryn is added or withdrawn. To avoid excessive or insufficient anticoagulation, regularly perform coagulation tests suitable for the anticoagulant used, at close intervals, especially in the first hours following the start or withdrawal of ATryn and monitor patients for bleeding or thrombosis. (5.2)

ADVERSE REACTIONS

Most common adverse reactions reported in clinical trials at a frequency of ≥ 5% were hemorrhage and infusion site reaction. (6)

To report SUSPECTED ADVERSE REACTIONS, contact Ovation Pharmaceuticals at 1-800-455-1141 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- ATryn enhances anticoagulant effect of heparin and low molecular weight heparin. (7)
- The half-life of ATryn may be altered by concomitant treatment with anticoagulants that use antithrombin to exert their anticoagulant effect. (7)

USE IN SPECIFIC POPULATIONS

- Pregnancy Category C. Studies in pregnant women have not shown that ATryn increases the risk of fetal abnormalities if administered during the third trimester of pregnancy. No data is available for use of ATryn in earlier stages of pregnancy.(8.1)

- Labor and Delivery: ATryn is used in the treatment of peri-partum women with hereditary antithrombin deficiency. (8.2)

- Nursing Mothers: ATryn administered by infusion will be present in breast milk at estimated concentrations 1/50 to 1/100 that of concentration in blood. Use only if clearly needed. (8.3)

See 17 for Patient Counseling Information.

Date: XX/XX/2009

FULL PRESCRIBING INFORMATION: CONTENTS*

1 INDICATIONS AND USAGE
2 DOSAGE AND ADMINISTRATION
- 2.1 Preparation for Administration
- 2.2 Recommended Dose and Schedule

3 DOSAGE FORMS AND STRENGTHS
4 CONTRAINDICATIONS
5 WARNINGS AND PRECAUTIONS
- 5.1 Hypersensitivity Reactions
- 5.2 Coagulation Monitoring Tests

6 ADVERSE REACTIONS
- 6.1 Clinical Trial Experience

7 DRUG INTERACTIONS
8 USE IN SPECIFIC POPULATIONS
- 8.1 Pregnancy
- 8.2 Labor and Delivery
- 8.3 Nursing Mothers
- 8.4 Pediatric Use
- 8.5 Geriatric Use

11 DESCRIPTION
12 CLINICAL PHARMACOLOGY
- 12.1 Mechanism of Action
- 12.2 Pharmacodynamics
- 12.3 Pharmacokinetics

13 NONCLINICAL TOXICOLOGY
- 13.1 Carcinogenesis, Mutagenesis, and Impairment of Fertility
- 13.2 Animal Toxicology and/or Pharmacology

14 CLINICAL STUDIES
15 REFERENCES
16 HOW SUPPLIED/STORAGE AND HANDLING
17 PATIENT COUNSELING INFORMATION

* Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

ATryn® is a recombinant antithrombin indicated for the *prevention* of peri-operative and peri-partum thromboembolic events in hereditary antithrombin deficient patients[1].

It is not indicated for *treatment* of thromboembolic events in hereditary antithrombin deficient patients.

2 DOSAGE AND ADMINISTRATION

For Intravenous Use Only after Reconstitution

2.1 Preparation for Administration

- Bring vials to room temperature no more than 3 hours prior to reconstitution.
- Reconstitute with 10 mL Sterile Water for Injection [(WFI) not supplied with ATryn] immediately prior to use. **Do not shake.**
- Do not use solution containing visible particulates or if it is discolored or cloudy.
- Draw solution from one or more vials into a sterile disposable syringe for intravenous administration or add solution to an infusion bag containing 0.9% sterile sodium chloride for injection (e.g., dilute solution to obtain a concentration of 100 IU/mL).
- Administer using an infusion set with a 0.22 micron pore-size, in-line filter.
- Administer contents of infusion syringes or diluted solution within 8 to 12 hours of preparation when stored at room temperature (68-77°F (20-25°C)).
- Discard unused product in accordance with local requirements.

2.2 Recommended Dose and Schedule

- The dosage of ATryn is to be individualized based on the patient's pre-treatment functional AT activity level (expressed in percent of normal) and body weight (expressed in kilograms) and using therapeutic drug monitoring (Table 1).
- The goal of treatment is to restore and maintain functional antithrombin (AT) activity levels between 80% - 120% of normal (0.8 - 1.2 IU/mL).

- Treatment should be initiated prior to delivery or approximately 24 hours prior to surgery to ensure that the plasma antithrombin level is in the target range at that time.
- Different dosing formulae are used for the treatment of surgical and pregnant patients. Pregnant women who need a surgical procedure other than Cesarean section should be treated according to the dosing formulae for pregnant patients.
- Administer loading dose as a 15-minute intravenous infusion, immediately followed by a continuous infusion of the maintenance dose.
- AT activity monitoring and dose adjustments should be made according to Table 2.
- Continue treatment until adequate follow-on anticoagulation is established.

Table 1: Dosing Formula for Surgical Patients and Pregnant Women

	Loading Dose (IU)		Maintenance Dose (IU/hour)	
Surgical Patients	$\frac{(100 - \text{baseline AT activity level})}{2.3}$	x Body Weight (kg)	$\frac{(100 - \text{baseline AT activity level})}{10.2}$	x Body Weight (kg)
Pregnant Women	$\frac{(100 - \text{baseline AT activity level})}{1.3}$	x Body Weight (kg)	$\frac{(100 - \text{baseline AT activity level})}{5.4}$	x Body Weight (kg)

AT Activity Monitoring and Dose Adjustment

AT activity monitoring is required for proper treatment. Check AT activity once or twice per day with dose adjustments made according to Table 2.

Table 2: AT Activity Monitoring and Dose Adjustment

Initial Monitor Time	AT Level	Dose Adjustment	Recheck AT Level
2 hours after initiation of treatment	< 80%	Increase 30%	2 hours after each dose adjustment
	80% to 120%	None	6 hours after initiation of treatment or dose adjustment
	> 120%	Decrease 30%	2 hours after each dose adjustment

As surgery or delivery may rapidly decrease the AT activity levels, check the AT level just after surgery or delivery. If AT activity level is below 80%, an additional bolus dose may be administered to rapidly restore decreased AT activity level. In such instances, the loading dose formulae in Table 1 should be used, utilizing in the calculation the last available AT activity result. Thereafter, restart the maintenance dose at the same rate of infusion as before the bolus.

3 DOSAGE FORMS AND STRENGTHS

ATryn is a sterile lyophilized formulation. Each vial of ATryn contains the potency stated on the label, which is approximately 1750 IU.

4 CONTRAINDICATIONS

ATryn is contraindicated in patients with known hypersensitivity to goat and goat milk proteins.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Allergic-type hypersensitivity reactions are possible. Patients must be closely monitored and carefully observed for any symptoms throughout the infusion period. Patients should be informed of the early signs of hypersensitivity reactions including hives, generalized urticaria, tightness of the chest, wheezing, hypotension, and anaphylaxis. If these symptoms occur during administration, treatment must be discontinued immediately and emergency treatment should be administered.

5.2 Coagulation Monitoring Tests

The anticoagulant effect of drugs that use antithrombin to exert their anticoagulation may be altered when ATryn is added or withdrawn. To avoid excessive or insufficient anticoagulation, coagulation tests suitable for the anticoagulant used (e.g., aPTT and anti-Factor Xa activity) are to be performed regularly, at close intervals, and in particular in the first hours following the start or withdrawal of ATryn. Additionally, monitor the patients for the occurrence of bleeding or thrombosis in such situation.

6 ADVERSE REACTIONS

The serious adverse reaction that has been reported in clinical studies is hemorrhage (intra-abdominal, hemarthrosis and post procedural). The most common adverse events reported in clinical trials at a frequency of ≥ 5% are hemorrhage and infusion site reaction.

6.1 Clinical Trial Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Adverse reactions that occurred in clinical trials with hereditary AT deficient patients are shown in Table 3 by System Organ Class.

Table 3: Adverse Reactions in Hereditary AT Deficient Patients (one event per patient, 2% of total population, n=47)

Gastrointestinal Disorders
Intra-abdominal Hemorrhage
General Disorders and Administration Site Disorders
Application Site Pruritus
Feeling Hot
Non-cardiac Chest Pain
Investigations
Hepatic Enzyme Abnormal
Musculoskeletal and Connective Tissue Disorders
Hemarthrosis
Renal and Urinary Disorders
Hematuria
Vascular Disorders
Hematoma

Immunogenicity

For ATryn, a potential safety issue is the development of an immunological reaction to the recombinant protein or any of the potential contaminating proteins. Assays were developed and used to detect antibodies directed against antithrombin (Recombinant), goat AT, or goat-milk proteins. No confirmed specific immunological reaction was seen in any of the patients tested, nor were there any clinical adverse events that might indicate such a response.

A post-marketing patient registry has been established to collect additional data on the immunogenic potential of ATryn in patients treated with ATryn on more than one occasion. Physicians are encouraged to participate in the registry by collecting pre- and post-treatment serum samples from patients according to instructions provided by Ovation Pharmaceuticals, Inc. and submitting them to Ovation for analysis for the development of antibodies to antithrombin (Recombinant). Serum samples should be collected within one week before initiation of treatment and on days 1, 7 and 28 days from initiation of treatment. Physicians wanting to participate in this program are encouraged to contact Ovation Pharmaceuticals, Inc. at 1-800-455-1141. Ovation will provide detailed instructions for the collection, processing and shipping of samples, as well as all tubes and labels that are necessary for the collection and processing of samples.

7 DRUG INTERACTIONS

The anticoagulant effect of heparin and low molecular weight heparin (LMWH) is enhanced by antithrombin. The half-life of antithrombin may be altered by concomitant treatment with these anticoagulants due to an altered antithrombin turnover. Thus, concurrent administration of antithrombin with heparin, low molecular

weight heparin, or other anticoagulants that use antithrombin to exert their anticoagulant effect must be monitored clinically and biologically. To avoid excessive anticoagulation, regular coagulation tests (aPTT, and where appropriate, anti-Factor Xa activity) are to be performed at close intervals, with adjustment in dosage of the anticoagulant as necessary.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C: In rats, a dose of 210 mg/kg/day ATryn (5-6 times the human dose for pregnant women) administered during most of the pregnancy and entire lactation showed a slight but statistically significant increase in pup mortality in day one through day four when compared to concurrent control (90% compared to 94% viability index for 210 mg/kg/day versus control). This slight statistical difference does not reflect a true treatment-related effect. This same dose was shown to be safe in a second rat study when administered around parturition and during lactation where the no adverse effect level for dam and pups was 210 mg/kg/day.

There are no adequate and well-controlled studies in pregnant women. Because animal reproductive studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Studies in pregnant women have not shown that ATryn increases the risk of fetal abnormalities if administered during the third trimester of pregnancy. In clinical trials in hereditary AT deficient patients, 22 pregnant women have been treated with ATryn around parturition.

No adverse reactions were reported in 22 neonates born from pregnant women treated with ATryn during clinical trials.

8.2 Labor and Delivery

ATryn is indicated for the treatment of pregnant women during the peri-partum period. Pregnant patients who need a surgical procedure other than Cesarean section are to be treated according to the dosing formulae for pregnant patients.

8.3 Nursing Mothers

ATryn will be present in breast milk at levels estimated to be 1/50 to 1/100 of its concentration in the blood. This level is the same as that estimated to be present in breast milk of normal lactating women which is not known to be harmful to breastfed neonates. However, caution should be exercised when ATryn is administered to a nursing woman. Use only if clearly needed.

In 2 reproductive toxicology studies performed in rats, antithrombin (Recombinant) was administered to pregnant dams at doses up to 210 mg/kg/day, resulting in

supraphysiologic plasma levels of antithrombin. Pups were allowed to breastfeed and were monitored for changes in prothrombin (PT) or aPTT, as well as pup viability, body weight at birth, growth, and development. In these studies, there were no adverse effects in offspring who consumed milk from dams treated with ATryn.

8.4 Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

8.5 Geriatric Use

Clinical studies of ATryn did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. In general, dose selection for an elderly patient should be cautious, usually starting at the low end of the dosing range, reflecting the greater frequency of decreased hepatic, renal, or cardiac function, and of concomitant disease or other drug therapy.

11 DESCRIPTION

ATryn for Injection is a nanofiltered, sterile, terminally heat treated, lyophilized dosage form. Antithrombin (Recombinant), active ingredient of ATryn, is a recombinant human antithrombin. It is a 432 amino acid glycoprotein with a molecular weight of approximately 57,215 Daltons. The molecular formula is: $C_{2191}H_{3457}N_{583}O_{656}S_{18}$. Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk. The goats in which antithrombin (Recombinant) is produced are USDA certified scrapie-free, and controlled for specific pathogens.

The amino acid sequence of Antithrombin (Recombinant) is identical to that of human plasma-derived antithrombin. Antithrombin (Recombinant) and plasma-derived antithrombin both contain six cysteine residues forming three disulphide bridges and 3-4 N-linked carbohydrate moieties. The glycosylation profile of Antithrombin (Recombinant) is different from plasma derived antithrombin, which results in an increased heparin affinity. When assayed in the presence of excess of heparin the potency of the recombinant product is not different from that of plasma derived product.

Each vial of ATryn is tested for potency stated on the product label using a reference standard calibrated against the World Health Organization international standard for antithrombin concentrate. In addition to Antithrombin (Recombinant), each vial of the product contains 100 mg glycine, 79 mg sodium chloride, and 26 mg sodium citrate. When reconstituted with 10 mL Sterile Water for Injection, the pH is approximately 7.0. Following reconstitution, the solution may be further diluted into 0.9 % sodium chloride for injection.

ATryn does not contain any preservatives nor is it formulated with human plasma proteins. Antithrombin (Recombinant) is affinity purified using a heparin immobilized resin and contains no detectable heparin (<0.0002 IU heparin per IU antithrombin) in the final product.

The purification and drug product manufacturing processes have been validated to demonstrate its capacity for removal and/or inactivation of viruses[4]. Results of removal and/or inactivation for each of the steps are shown in Table 4.

Table 4: Viral Clearance Results (log_{10} reductions)

Process Step	Pseudorabies Virus	Xenotropic Murine Retrovirus	Human Adenovirus	Porcine Parvovirus
Tangential Flow Filtration	≥5.1			
Affinity Chromatography	1.6	1.2	NA	1.4
Nanofiltration		≥3.8	≥6.3	≥3.7
Ion Exchange Chromatography	3.6	1.0	≥7.1	NA
Hydrophobic Interaction Chromatography	≥5.6	≥4.4	≥4.8	≥5.7
Heat Treatment	2.8	≥5.0	≥1.8	2.4
Total Reduction	**≥18.7**	**≥15.4**	**≥20.0**	**≥13.2**

NA = Not Applicable since log_{10} reduction was less than 1.0.

In addition, although the goats are from a closed, USDA certified scrapie-free herd, the purification process was challenged to remove prions. The manufacturing steps were shown capable of achieving the following log_{10} reductions: 2.0 (tangential filtration), 2.2 (affinity column), ≥ 3.3 (ion exchange column), ≥ 3.8 (hydrophobic interaction column).

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Antithrombin (AT) plays a central role in the regulation of hemostasis. AT is the principal inhibitor of thrombin and Factor Xa[5], the serine proteases that play pivotal roles in blood coagulation. AT neutralizes the activity of thrombin and Factor Xa by forming a complex which is rapidly removed from the circulation. The ability of antithrombin to inhibit thrombin and Factor Xa can be enhanced by greater than 300 to 1000 fold when AT is bound to heparin.

12.2 Pharmacodynamics

Hereditary AT deficiency causes an increased risk of venous thromboembolism (VTE). During high-risk situations such as surgery or trauma or for pregnant women, during the peri-partum period, the risk of development of VTEs as compared to the normal population in these situations is increased by a factor 10 to 50[6,7].

In hereditary antithrombin deficient patients ATryn restores (normalize) plasma AT activity levels during peri-operative and peri-partum periods.

12.3 Pharmacokinetics

In an open-label, single dose pharmacokinetic study, male and female patients (≥ 18 years of age) with hereditary AT deficiency, received either 50 (n = 9, all females) or 100 (n = 6, 2 males and 4 females) IU/kg ATryn intravenously. These patients were not in high-risk situations. The baseline corrected pharmacokinetic parameters for antithrombin (Recombinant) are summarized in Table 5.

Table 5: Baseline Corrected Mean Pharmacokinetic Parameters (%CV)

Parameter	50 IU/kg	100 IU/kg
CL (mL/hr/kg)	9.6 (34.4)	7.2 (15.3)
Half-life (hrs)	11.6 (84.7)	17.7 (60.9)
MRT (hrs)	16.2 (74.9)	20.5 (40.2)
Vss (mL/kg)	126.2 (37.4)	156.1 (43.4)

Incremental recovery [mean (%CV)] was 2.24 (20.2) and 1.94 (14.8) %/IU/kg body weight for 50 and 100 IU/kg, respectively.

Population pharmacokinetic analysis of hereditary deficient patients in a high risk situation revealed that the clearance and volume of distribution in pregnant patients were (1.38 L/h and 14.3 L respectively) which are higher than non-pregnant patients (0.67 L/h and 7.7 L respectively). Therefore, distinct dosing formulae for surgical and pregnant patients should be used (see 2.2, Recommended Dose and Schedule).

As compared to plasma derived antithrombin, ATryn has a shorter half-life and more rapid clearance (approximately nine and seven times, respectively).

Pharmacokinetics may be influenced by concomitant heparin administration, as well as surgical procedures, delivery, or bleeding. AT activity monitoring (see 2.2 Recommended Dose and Schedule) should be performed to properly treat such patients.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, and Impairment of Fertility

Carcinogenesis: No carcinogenicity data for ATryn are available in animals or humans.

Mutagenesis and Genotoxicity: ATryn was not mutagenic when tested in the Ames bacterial test and in *in vitro* cytogenetic assays nor was it shown to be genotoxic when tested in an *in vivo* test to assess chromosomal aberration.

Impairment of Fertility: No studies have been conducted to evaluate the effects of ATryn on fertility in humans.

13.2 Animal Toxicology and/or Pharmacology

Pharmacokinetic and toxicokinetic (1 single, 2 repeated dose) studies of antithrombin (Recombinant) were performed in mice, rats, dogs and monkeys. In toxicokinetic studies in monkeys the area under the curve was 3-4 times greater than in the rat at all doses used.

The toxicological profile of antithrombin (Recombinant) administered by the intravenous route as bolus injections and infusions has been evaluated in both single- and repeat-dose studies performed in rats, dogs, and monkeys across a range of doses from 2.1 to 360 mg/kg. The highest doses in the single dose toxicity studies in rats and dogs were 360 mg/kg and 210 mg/kg, respectively. Toxicities observed were limited to transient injection site swelling observed in rats and dogs at the highest doses tested, and increased AST at highest dose in the dog study, both resolved during recovery period.

The highest dose in the 28-day repeated-dose toxicity study in rats was 360 mg/kg/day. The toxicity at this dose was limited to transient limb swelling and local injection site bruising and swelling. The highest dose in the 14-day repeated-dose toxicity study in monkeys was 300 mg/kg/day or approximately 7-8 times human dose. Toxicities observed in female monkeys at this dose included internal bleeding, hematological changes and liver toxicity, with one out of three female animals showing multifocal hepatic necrosis. Both sexes showed increased AST and ALK on day 15, with both parameters returning to normal by day 22. There was no adverse effect in monkeys dosed with 120 mg/kg/day.

14 CLINICAL STUDIES

The efficacy of ATryn to prevent the occurrence of venous thromboembolic events was assessed by comparing the incidence of the occurrence of such events in 31 ATryn treated hereditary AT deficient patients with the incidence in 35 human plasma-derived AT treated hereditary AT deficient patients. Data on ATryn-treated patients were derived from two prospective, single-arm, open-label studies. Data on plasma AT treated patients were collected from a prospectively designed concurrently conducted retrospective chart review. Patients in both studies had confirmed hereditary AT deficiency (AT activity ≤ 60% of normal) and a personal history of thromboembolic events. Patients had to be treated in the peri-operative and peri-partum period. ATryn was administered as a continuous infusion for at least 3 days, starting one day prior to the surgery or delivery. Plasma AT was administered for at least two days as single bolus infusions. Due to the retrospective nature of the study, dosing was done with the locally available AT concentrate according to the local practice.

The occurrence of a venous thromboembolic event was confirmed if signs and symptoms for such events were confirmed by a specific diagnostic assessment, or when treatment for an event was initiated based on diagnostic imaging, without the presence of signs and symptoms. The efficacy was assessed during treatment with AT and up to 7 days after stopping AT treatment.

In the ATryn-treated group there was one confirmed diagnosis of an acute deep vein thrombosis (DVT). The incidence of any thromboembolic event from the start of treatment to 7 days after last dosing is summarized by treatment group in Table 6 as are the Clopper-Pearson exact 95% CI for the proportion of patients with a thromboembolic event and the exact 95% lower confidence bound for the difference between treatments.

Table 6: Overall Incidence of Any Confirmed Thromboembolic Event

Plasma AT				ATryn				Lower 95% Confidence Bound of Difference
No. of Pts. Assessed	No. of Pts. with Events	% of Pts. with Events	95% CI*	No. of Pts. Assessed	No. of Pts. with Events	% of Pts. with Events	95% CI	
35	0	0.0	0.00, 10.00	31	1	3.2	0.08, 16.70	-0.167

* The 95% confidence intervals were calculated using Clopper-Pearson methodology.
AT=Antithrombin; No.=Number; Pts.=Patients; CI=Confidence Interval

The lower 95% confidence bound of difference between treatment groups was -0.167, a value that is greater than the pre-specified lower confidence bound of -0.20. This demonstrates that ATryn was non-inferior to plasma AT in terms of the prevention of peri-operative or peri-partum thromboembolic events.

Supportive data come from a study in the same population with 5 hereditary AT deficient patients treated on 6 occasions in a compassionate use program and provides additional reassurance of the efficacy of ATryn. None of these patients reported a thromboembolic event.[2]

15 REFERENCES

(1) Patnaik MM, Moll S. Inherited antithrombin deficiency: a review. *Haemophilia* 2008;14:1229-39.

(2) Konkle BA, Bauer KA, Weinstein R, Greist A, Holmes HE, Bonfiglio J. Use of recombinant human antithrombin in patients with congenital antithrombin deficiency undergoing surgical procedures. *Transfusion* 2003 March;43(3):390-4.

(3) Edmunds T, Van Patten SM, Pollock J et al. Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. *Blood* 1998 June 15;91(12):4561-71.

(4) Echelard Y, Meade H, Ziomek C. The first biopharmaceutical from transgenic animals: ATryn. *Modern Biopharmaceuticals* 2005;995-1016.

(5) Maclean PS, Tait RC. Hereditary and acquired antithrombin deficiency: epidemiology, pathogenesis and treatment options. *Drugs* 2007;67(10):1429-40.

(6) Buchanan GS, Rodgers GM, Ware Branch. The inherited thrombophilias: genetics, epidemiology, and laboratory evaluation. *Best Pract Res Clin Obstet Gynaecol* 2003 June;17(3):397-411.

(7) Walker ID, Greaves M, Preston FE. Investigation and management of heritable thrombophilia. *Br J Haematol* 2001;114:512-28.

16 HOW SUPPLIED/STORAGE AND HANDLING

Dosage Form

NDC 67386-521-51

Approximately 1750 IU/vial in a sterile white to off-white lyophilized powder for reconstitution.Each carton contains one single dose vial of ATryn.

The actual potency of ATryn is stated on the vial label and carton.

Storage and Handling

Store ATryn refrigerated at between 2-8°C (36-46°F).

Do not use product beyond the expiration date printed on the package. Discard unused portions.

17 PATIENT COUNSELING INFORMATION

Inform patients that allergic-type hypersensitivity reactions are possible and instruct them to inform their physicians about any past or present known hypersensitivity to goats or goat milk proteins prior to treatment with ATryn. Inform patients of the early signs of hypersensitivity reactions including hives, generalized urticaria, tightness of the chest, wheezing, hypotension, and anaphylaxis and to notify their health care provider immediately if these events develop.

Inform patients about the risk of bleeding when ATryn is administered with other anticoagulants and instruct them to notify their physicians of any bleeding events while on treatment with ATryn.

Manufacturer:
GTC Biotherapeutics, Inc.
Framingham, MA 01702, U.S.A.

U.S. License No. XXXX

Marketed by:
Ovation Pharmaceuticals, Inc.
Deerfield, IL 60015, U.S.A.

Issued: February 2009

Component # XXXX

F

Transgenically Produced Human Antithrombin: Structural and Functional Comparison to Human Plasma–Derived Antithrombin

By Tim Edmunds, Scott M. Van Patten, Julie Pollock, Eric Hanson, Richard Bernasconi, Elizabeth Higgins, Partha Manavalan, Carol Ziomek, Harry Meade, John M. McPherson, and Edward S. Cole

Recombinant human antithrombin (rhAT) produced in transgenic goat milk was purified to greater than 99%. The specific activity of the rhAT was identical to human plasma-derived AT (phAT) in an in vitro thrombin inhibition assay. However, rhAT had a fourfold higher affinity for heparin than phAT. The rhAT was analyzed and compared with phAT by reverse phase high-performance liquid chromatography, circular dichroism, fluorophore-assisted carbohydrate electrophoresis (FACE), amino acid sequence, and liquid chromatography/mass spectrography peptide mapping. Based on these analyses, rhAT was determined to be structurally identical to phAT except for differences in glycosylation. Oligomannose structures were found on the Asn 155 site of the transgenic protein, whereas only complex structures were observed on the plasma protein. RhAT contained a GalNAc for galactose substitution on some N-linked oligosaccharides, as well as a high degree of fucosylation. RhAT was less sialylated than phAT and contained both N-acetylneuraminic and N-glycolylneuraminic acid. We postulate that the increase in affinity for heparin found with rhAT resulted from the presence of oligomannose-type structures on the Asn 155 glycosylation site and differences in sialylation.

© 1998 by The American Society of Hematology.

ANTITHROMBIN (AT) is a serine protease inhibitor that inhibits thrombin and factor Xa and, to a lesser extent, factors IXa, XIa, XIIa, tPA, urokinase, trypsin, plasmin, and kallikrein.[1-4] Human AT, which is synthesized in the liver, is normally present in plasma at levels of 14 to 20 mg/dL.[5,6] It has a molecular weight of approximately 58,000 Da and contains 432 amino acids, three disulfide bridges, and four carbohydrate side chains, which account for 15% of the total mass.[7,8] Decreased levels of AT may be found in the serum of individuals who have either a hereditary deficiency of AT or an acquired deficiency, which can result from a number of pathological conditions.[3]

AT circulates in a form with low inhibitory activity.[9,10] The addition of heparin increases the inhibitory capacity of AT for thrombin by 1,000- to 5,000-fold or greater[11-13] The AT binds to a well defined pentasaccharide on the heparin chain.[13-16] The interaction between AT and thrombin then produces a tightly bound TAT (thrombin:antithrombin) complex[17] that is essentially irreversible[18] and cleared quickly from circulation. Therapeutic use of heparin as an anticoagulant works through activation of endogenous AT. Patients whose AT levels are very low can become refractory to heparin therapy.[19,20] Although circulating AT provides an anticoagulant effect with exogenously administered heparin, the heparan sulfate glycosaminoglycans on the endothelial layer lining the blood vessels may be the physiological site of action for circulating AT.[21-24]

We have previously shown that human proteins can be made in the milk of transgenic goats[25,26] and that transgenic production of large quantities of recombinant protein is feasible.[27] We have now produced recombinant human antithrombin (rhAT) in this production mode focusing on large-scale production. The current yearly production of plasma-derived AT is estimated at approximately 100 kg worldwide. Because AT is glycosylated[7] and glycosylation is important for half-life and sometimes function[28] of glycoproteins, a mammalian expression system was favored for recombinant production of this glycoprotein for therapeutic use. Assuming the highest level of recombinant protein production in mammalian cell culture is 100 mg/L of culture media and 50% recovery through the purification process, 2,000,000 L of culture media would be required to produce 100 kg of purified rhAT. At 2 g/L expression level in goat's milk, 100,000 L of milk from approximately 150 goats would be sufficient, assuming similar recovery. We present here the initial characterization of human AT transgenically produced in the milk of goats (rhAT) and its comparison with human plasma–derived AT (phAT).

MATERIALS AND METHODS

Production of the transgenic animal. Transgenic goats expressing rhAT in their milk, under control of the β-casein promoter, were made essentially as described previously.[27] The goats were developed in collaboration with Dr Karl Ebert of Tuft's University School of Veterinary Medicine (Boston, MA). The goat β-casein gene was cloned as previously described by Roberts et al.[29] The coding region between exons 2 and 7 was removed and replaced with an *Xho* I restriction site.[27,30] The human AT cDNA was derived from a modified pBAT6 plasmid,[31] which allows excision of the cDNA as an *Xho* I to *Sal* I fragment. The *Xho* I/*Sal* I human AT cDNA was ligated between exons 2 and 7 of the goat β-casein gene to form a goat β-casein–human AT cDNA transgene.

Embryos were collected, microinjected, and transferred to recipient female goats as previously described.[27] A founder (F_0) transgenic goat, a male was identified by analyzing genomic DNA from both a sample of ear tissue and blood by polymerase chain reaction (PCR)[32] and by Southern blot analysis.[27] This founder male was bred to nontransgenic females and produced transgenic female and male offspring. Transmission of the transgene was analyzed in genomic DNA isolated from blood by PCR and Southern blot analysis. The milk from two of the (F_1) transgenic female goats was pooled and used in this study.

Purification. Milk from AT transgenic goats was stored frozen at −40°C before use. For purification, milk was thawed, proprietary chemicals were added to enhance filtration, and the milk was clarified through a 500-kD tangential flow membrane filtration unit (AG Technology Corp, Needham, MA). The permeate from the filter was

From the Cell and Protein Therapeutics Department, Genzyme Corp, and Genzyme Transgenics Corp, Framingham, MA.

Submitted July 9, 1997; accepted January 5, 1998.

Address reprint requests to Edward S. Cole, PhD, Cell and Protein Therapeutics Department, Genzyme Corp, 1 Mountain Rd, Framingham, MA 01701-9322.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*

© 1998 by The American Society of Hematology.

0006-4971/98/9112-0020$3.00/0

loaded directly onto a Heparin Hyper-D (Biosepra, Marlborough, MA) affinity chromatography column. The column was equilibrated in 40 mmol/L sodium phosphate, 135 mmol/L NaCl, pH 6.9, washed with 20 mmol/L sodium phosphate, 400 mmol/L NaCl, pH 7.0 and eluted with 20 mmol/L sodium phosphate, 2.5 mol/L NaCl, pH 7.0.

The heparin column eluate was concentrated and diafiltered against 20 mmol/L sodium phosphate, 65 mmol/L NaCl, pH 7.0 by ultrafiltration to reduce the conductivity. This solution was applied to an ANX Sepharose Fast Flow column (Pharmacia, Piscataway, NJ) equilibrated with 20 mmol/L sodium phosphate, 65 mmol/L NaCl, pH 7.0. The column was washed with equilibration buffer and eluted with 20 mmol/L sodium phosphate, 320 mmol/L NaCl, pH 6.7. The anion exchange eluate was adjusted to 1.26 mol/L citrate by addition of sodium citrate and loaded onto a Methyl HyperD column (Biosepra) equilibrated with 1.26 mol/L sodium citrate, 7.7 mmol/L citric acid, pH 7.0. The column was washed with equilibration buffer and eluted with 0.9 mol/L sodium citrate, 5.5 mmol/L citric acid, pH 7.0. The rhAT was then concentrated to 25 mg/mL by tangential flow ultrafiltration and formulated.

Analytical methods. A commercially available (Thrombate; Miles Inc, Elkart, IN) preparation of human plasma AT was used for comparison with rhAT. The lyophilized product (Lot # 03B002 B) was reconstituted with 10 mL of high-performance liquid chromatography (HPLC) grade water and aliquots (53.3 IU/mL) frozen at −80°C. Goat plasma AT was isolated from goat plasma by an adaptation of the methods shown for the recombinant AT.

The expression level of the AT in the milk was determined by using a two-stage colorimetric endpoint assay in a microplate format, which measures the degree of thrombin inhibition. This assay is a modified AT/heparin cofactor assay.[33,34] In this method, AT was added to an excess of porcine heparin (Grade I-A #H3393; Sigma, St Louis, MO) followed by the addition of a constant amount of thrombin. After a period of incubation, thrombin activity was measured by the addition of a thrombin-specific chromogenic substrate (S2238, Chromogenics, Mölndal, Sweden).

Inhibitory activity of purified rhAT and phAT was measured with respect to thrombin in a similar manner by using the S2238 substrate and an excess of heparin (55.5 nmol/L). The concentration of thrombin (Calbiochem #605190, La Jolla, CA) was 243 mU/mL for this assay, with a substrate concentration of 337 μmol/L. Inhibitory activity with respect to factor Xa was measured in an equivalent assay, but with the substitution of factor Xa (Calbiochem #233526; 6.46 mU/mL in the assay) for thrombin, and using the S2765 substrate from Kabi. In both assays the enzyme was added to a mixture of AT and heparin for a 10-minute preincubation at 37°C before the initiation of the reaction by addition of substrate. Reaction volumes were 0.6 mL, and all reactions were stopped after a period of 10 minutes by the addition of glacial acetic acid. The reaction rate was shown to be linear for this 10-minute period. Heparin cofactor activation of thrombin inhibition was measured using multiple subsaturating concentrations of heparin in the assays with AT at 3 nmol/L.

Total protein was determined by using a modified Bradford protein assay (Pierce, Rockford, IL) with bovine serum albumin as standard. The concentration of the purified protein was confirmed by amino acid analysis as previously described.[35] Recovery of rhAT after each purification step was determined by a rapid reverse-phase HPLC (RP-HPLC) chromatography method performed by using a Hewlett Packard (Wilmington, DE) 1100 series HPLC equipped with detection at 214 nm. Samples were analyzed on a POROS R2/H column (2.1 × 30 mm; Perseptive Biosystems, Framingham, MA) equilibrated with 0.1% trifluoro acetic acid (TFA) in HPLC grade water at a flow rate of 2.0 mL/min. rhAT was eluted from the column by using 0.08% TFA in acetonitrile. Test sample concentrations were determined by rhAT peak area comparisons to a standard quantitated by amino acid analysis.

Polyacrylamide gel electrophoresis was performed according to the Laemmli method[36] by using gradient gels (10% to 20%) (Integrated Separations Systems, Natick, MA). Gels were stained with silver by the method of Morrissey.[37] Purity was determined by scanning the gels with an LKB Model 2202 laser densitometer (LKB Inst, Uppsala, Sweden). BIORAD molecular weight standards were used for molecular weight estimation. Western blot analysis was performed by using a modification of the method of Burnette.[38] The labeling antibody was affinity purified sheep anti-hAT-HRP (SeroTec, Oxford, UK). Development was with the enhanced chemiluminescence (ECL) system (Amersham, Princeton, NJ).

Protein purity was assessed by RP-HPLC on a Hewlett Packard 1090 HPLC equipped with photodiode array detection. Samples were diluted to 80 μg/mL in 0.1% TFA and 250 μL aliquots analyzed on a Vydac C4 column (2.1 × 250 mm) equilibrated in 0.1% TFA. The column was developed with a complex gradient at a flow rate of 0.3 mL/min. Solvent A was 0.1% TFA and solvent B was 0.08% TFA in acetonitrile; 0 to 2 minutes = 0% solvent B; 2 to 22 minutes = 0% to 40% solvent B; 22 to 27 minutes = 40% to 45% solvent B; 27 to 34.5 minutes = 45% to 60% solvent B; 34.5 to 40 minutes = 60% to 90% solvent B; and 40 to 42 minutes = 90% solvent B. Peaks were integrated at 215 nm and a purity value was calculated.

For peptide mapping, samples of AT were reduced and pyridylethylated and the modified protein was desalted by RP-HPLC on a C4 column. Digestions of both the native and reduced/pyridylethylated protein were performed with Lys-C protease at pH 8.5 at an enzyme:substrate ratio of 1:50 for 18 hours at 37°C. Resultant digests were quenched and peptide mapping was performed on a Vydac C8 reverse phase column (2.1 × 150 mm). Peptides were eluted in an acetonitrile/TFA gradient.

For LC/MS analysis, eluant from the chromatography column was introduced directly into the electrospray interface of the mass spectrometer and spectra acquired over m/z range of 200 to 4,000 at a 3-second scan rate. Electrospray mass spectrometry was performed on a Finnigan TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan Electrospray source. Chromatography was performed on a Michrom UMA HPLC at a flow rate of 50 μL/min.

Amino terminal sequence analysis was performed by using an Applied Biosystems (Foster City, CA) 477 sequencer. Phenylthiohydantoin (PTH) amino acid analysis was performed with an on-line Applied Biosystems 120A PTH analyzer equipped with an Applied Biosystems 2.1 × 220-mm PTH C-18 column.

Monosaccharide analysis was performed according to the method of Hardy et al[39] by using a Dionex HPLC (Sunnyvale, CA) equipped with a pulsed amperometric detector. Sialic acid determination was performed by using the thiobarbituric acid method as modified by Powell and Hart[40] by using a Hewlett Packard 1090 HPLC. Monosaccharide standards were purchased from Phanstiel Laboratories Inc (Waukegan, IL).

Oligosaccharide content was examined by using the fluorophore-assisted carbohydrate electrophoresis (FACE) system (Glyko, Novato, CA) and LC/MS analysis of glycopeptides. FACE was performed on oligosaccharides released from glycoproteins by using N-Glycanase (Genzyme, Framingham, MA). Proteins were denatured with 0.15% sodium dodecyl sulfate (SDS), 65mmol/L β-mercaptoethanol at 100°C for 5 minutes. SDS binding was displaced with 1.2% Nonidet-40 after cooling on ice for 5 minutes. N-Glycanase was added at 150 U/mg AT and samples incubated overnight at 37°C. The released oligosaccharides were labeled overnight at 37°C with the fluorescent label 8, aminonaphthalene-1,3,6-trisulfonate (ANTS) by using reagents and protocol included in the Oligosaccharide Labelling Reagent Pack (Glyko). Labeled samples were loaded onto precast oligosaccharide profiling gels from Glyko. Gels were run at 15 mA/gel for 85 minutes. Gels were imaged by using an SE1000 Imager (Glyko) and quantitated by using FACE Analytical Software (Glyko).

Heparin binding of purified AT was assessed by fractionation on a TSK-Gel Heparin 5PW affinity column (5 mm internal diameter [ID] × 50 mm; TosoHaas, Montgomeryville, PA) on a Hewlett Packard 1050 HPLC. Samples were applied to the column equilibrated in 50 mmol/L Tris-Cl, 10 mmol/L sodium citrate, pH 7.4 at a flow rate of 0.5 mL/min. The column was washed for 10 minutes with equilibration buffer and eluted with a linear gradient of 0 to 3 mol/L NaCl in equilibration buffer over 20 minutes. Protein was monitored by absorbance at 215 nm.

Absorbance of a blank run was subtracted to give the final chromatograms.

Heparin binding of AT was also assessed by measuring the change in fluorescence of Trp according to Fan et al.[41] Samples of AT were diluted to 20 nmol/L in 20 mmol/L sodium phosphate, 100 mmol/L NaCl, 100 μmol/L EDTA, pH 7.4 containing 0.1% PEG-4000 (Fluka, Buchs, Switzerland). Fluorescence was measured on a Spex FluoroMax fluorometer (Spex Industries, Edison, NJ) by using an excitation wavelength of 280 nmol/L (5-nm bandwidth) and an emission wavelength of 340 nmol/L (10-nm bandwidth). Heparin (Sigma Grade I-A #H3393; porcine) was added sequentially in 1-μL aliquots to up to 16 μL total added volume.

Circular dichroism spectroscopy was performed on a Jasco J-720 spectropolarimeter (Jasco Inc, Easton, MD). The instrument was calibrated with (+)-10-camphorsulfonic acid. Measurements were made at room temperature by using cylindrical cuvettes with path lengths 0.01 cm (far ultraviolet, 170 to 260 nm) and 1.0 cm (near ultraviolet, 250 to 360 nm). Data analysis was performed on the average of at least 45 scans. The average spectrum was smoothed by Fourier transformation and corrected for the baseline. The sample was in 133 mmol/L glycine, 135 mmol/L NaCl, 10 mmol/L sodium citrate buffer at pH 7.0.

RESULTS

Expression of rhAT. A graphic presentation of rhAT expression in transgenic goat milk during a full lactation cycle is shown in Fig 1. The rhAT expression level remained relatively constant throughout the full lactation cycle, which lasted over 300 days with a fairly constant yield throughout the lactation. The purification process (see Materials and Methods) provided a cumulative yield of greater than 50% (Table 1) with high step recoveries at all stages during the process.

Purity. The purity of the rhAT was greater than 99% and was at least equivalent to that observed for the commercial phAT as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Fig 2A). No protein bands other than AT (determined by Western blot; Fig 2B) were evident with silver staining at a high protein load (Fig 2A). The higher molecular weight bands in all cases appeared to be multimers of AT with the major oligomeric form in the phAT having the apparent molecular weight of a dimer. Based on this gel, the rhAT has a lower level of oligomerization than the phAT. Purity as assessed by RP-HPLC was determined to be greater than 99% (Fig 3). The chromatographic profile and retention time of the rhAT was similar to the plasma-derived protein. Analysis of the leading shoulder areas (seen in both samples) by peptide

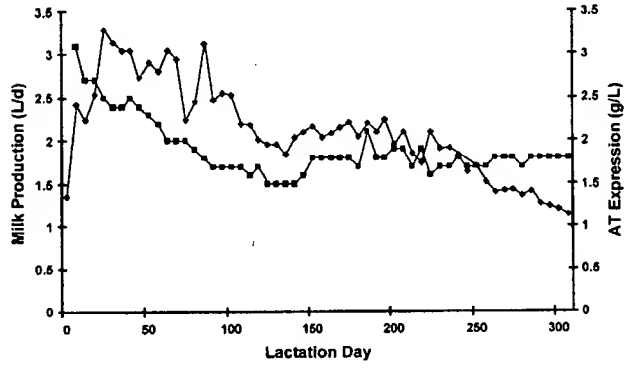


Fig 1. Milk production and rhAT expression level of a typical lactation cycle from one female goat. (◆), milk volume in L/d; (■), rhAT level in g/d.

Table 1. Compilation of the Percent Recovery for Each Step of Purification and Overall for the Process

Step	Total ATIII (g)	Step Recovery	Overall Yield
Diluted milk	115.0	—	100%
Heparin eluate	87.3	76%	76%
Ultrafiltration	86.0	98%	75%
DEAE eluate	84.7	98%	74%
Methyl eluate	67.3	80%	59%
UF/Formulation	61.0	91%	53%

Data represent the average of 3 runs at this scale.

mapping coupled with mass spectrometry, identified these peaks as AT molecules that are partially oxidized (data not shown).

Assessment of activity and heparin binding. The transgenically produced rhAT was found to have a specific activity of 6 IU/mg protein, equivalent to that of the phAT, by using an assay that measured inhibition of thrombin in an excess of heparin. The inhibitory capacity of rhAT and phAT in both thrombin and factor Xa activity assays was examined (Fig 4). Equivalent inhibition was observed with both the major known targets of AT.

Heparin cofactor activation of rhAT was examined by varying the amount of heparin used in inhibition assays of either thrombin or factor Xa (Fig 5). These assays showed that rhAT requires a lower concentration of heparin than phAT for inhibition of both enzymes. The heparin concentrations required for half-maximal inhibition of thrombin by rhAT and phAT were 0.045 nmol/L and 0.151 nmol/L, respectively. For inhibition of factor Xa, these values were 0.71 nmol/L and 2.22 nmol/L for rhAT and phAT. In both cases, rhAT required a threefold to fourfold lower concentration of heparin for activation of the inhibitor.

Analysis of the binding characteristics of each AT was also examined with a solid-phase heparin affinity column (Fig 6). The complexity observed in the elution profile (Fig 6A) was caused by both the heterogeneous population of heparin used to make the column and the presence of the higher affinity β-isoform of AT, which lacks glycosylation at Asn 135. The α isomer eluted as two peaks, at 21 and approximately 25 minutes for rhAT and 20 and approximately 24 minutes for phAT. The β-AT form also eluted as two peaks at 24 and 28.5 minutes for rhAT and 23 and 28 minutes for phAT. Both the major and minor peaks associated with rhAT required a higher salt concentration for elution compared with the coordinate peaks of phAT indicating a higher heparin affinity for all forms of the transgenically produced AT. Because the first β-isoform peak coeluted with the second α-isoform peak, quantitation of the forms from this method was not possible. Examination of the thrombin inhibitory activity across the elution pattern of the rhAT (Fig 6B) indicated that all of the fractions detected as having protein also had activity.

Examination of heparin binding affinity by using the Trp fluorescence assay[41] provided additional evidence that the two molecules differ in their affinity for heparin (Fig 7). A fourfold higher affinity for heparin was observed with the rhAT when compared with the phAT. The calculated binding constants (K_d values) were 10.3 nmol/L and 41.2 nmol/L for rhAT and phAT, respectively. However, the F_{max} (fluorescence at saturating

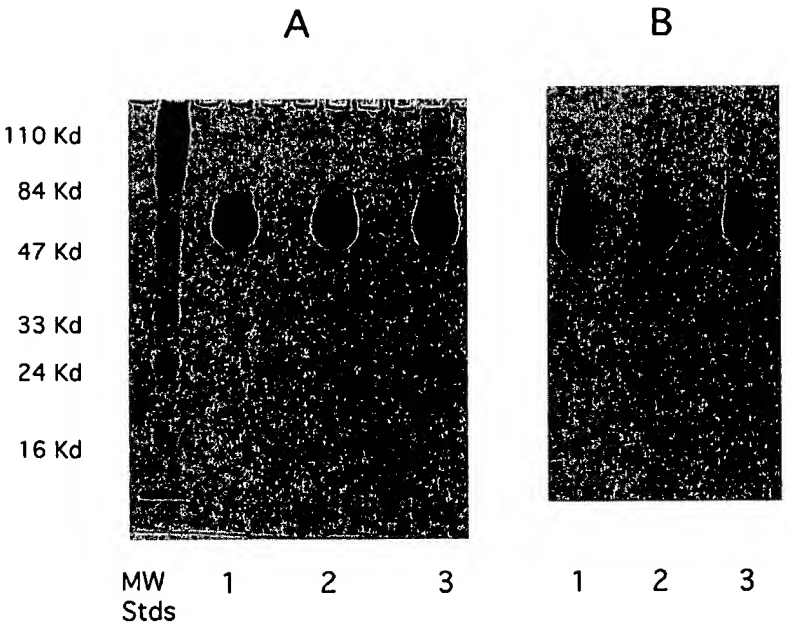


Fig 2. SDS-PAGE gel (A) and Western blot (B) of rhAT (lane 1), rhAT assay standard (lane 2), and phAT (lane 3). Molecular weight markers are shown on the silver stained gel (A). Twenty micrograms of protein were applied to each sample lane (lanes 1, 2, and 3). The SDS-PAGE gel (A) was developed with the Morrissey silver stain, and the Western blot (B) was developed with a sheep antihuman AT-HRP antibody (SeroTec) and color development was the ECL system (Amersham).

heparin) values were indistinguishable for rhAT and phAT. Dissociation constants were calculated by using nonlinear least squares analysis as described by Fan et al[41] assuming a mean molecular weight of 13,500 D for heparin.[42]

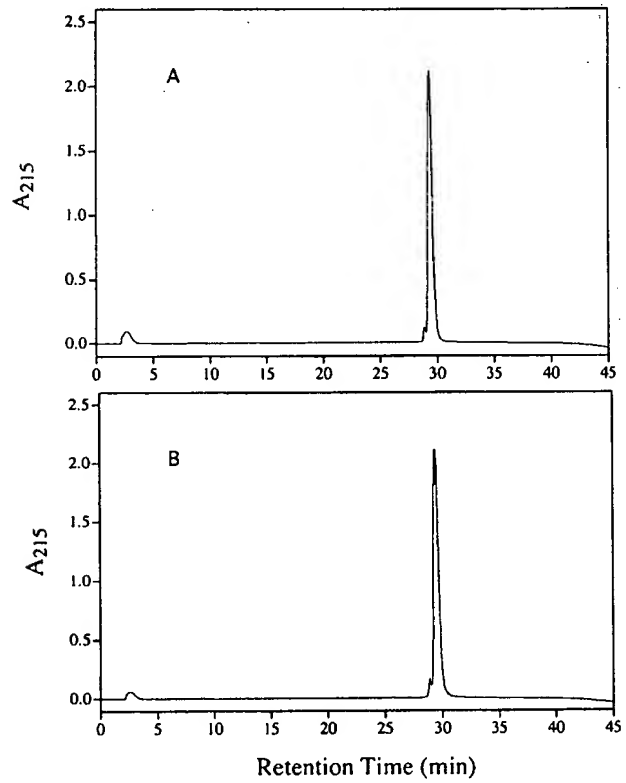


Fig 3. RP-HPLC chromatograms showing the purity of rhAT (A) and phAT (B). Chromatography was performed with 20 μg of protein applied to a Vydac C4 column (2.1 × 250 mm) with a series of linear gradients (see Materials and Methods). Detection was by absorbance at 215 nm.

Structural characterization. A detailed biochemical characterization was performed in an effort to determine the basis for the increased heparin affinity in the transgenic protein. N-terminal sequence analysis confirmed that the rhAT had the correct N-terminal sequence. A minor sequence lacking the first two residues and beginning with serine was observed in both the rhAT and phAT, with a slightly lower percentage (8% *v* 13%) of this form present in the rhAT. The reduced and pyridylethylated peptide map of rhAT was essentially identical to that of phAT (Fig 8). The only differences observed were in the regions of the three glycopeptides (K8, K15, and K18). The peptide containing the Asn 135 glycosylation site (K12) is a tripeptide and was not retained on the chromatographic column used. The differences observed in the glycopeptides were caused by the increased glycosylation heterogeneity present in the transgenic protein.

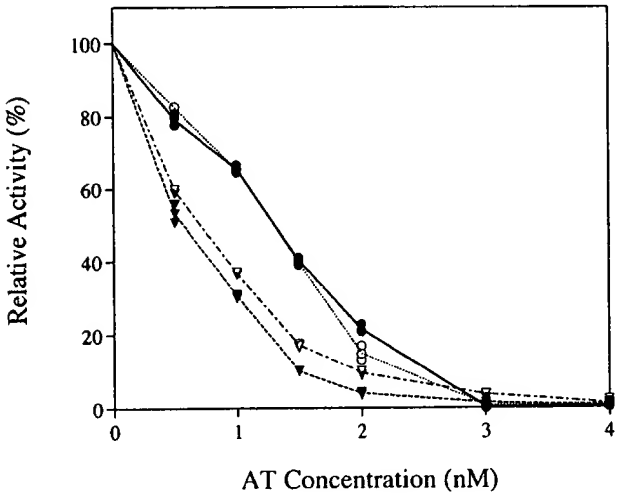


Fig 4. Comparative inhibition of thrombin (circles) and factor Xa (inverted triangles) by rhAT (● and ▼) and phAT (○ and ▽) with a saturating concentration of heparin.

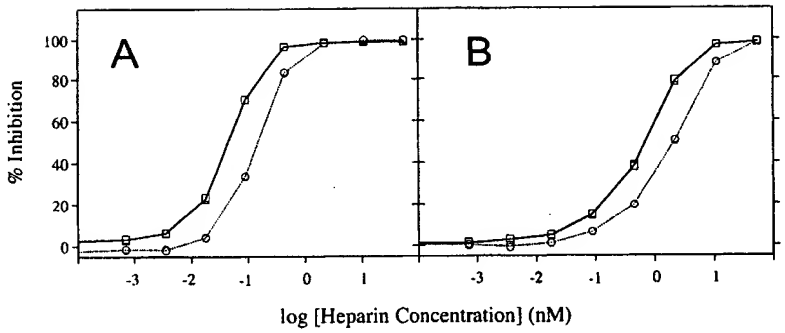


Fig 5. Heparin cofactor activation assays measured by using increasing concentrations of heparin. (A) Activation of thrombin inhibition. (B) Activation of factor Xa inhibition. (□), rhAT; (○), phAT.

The primary sequence of rhAT was confirmed by on-line LC/MS analysis of an endoproteinase Lys-C digest. By using this procedure, 27 of the 35 theoretical Lys-C peptides were identified (Table 2). Seven of the eight unidentified peptides had masses of less than 375 D, which would result in a doubly charged ion below the 200 mass/charge ratio (m/z) lower mass range used in these studies. The eighth peptide (K26) was observed as an incomplete cleavage product because of the presence of a proline C-terminal to the lysine. The only post-translational modifications detected were at the known N-glycosylation sites with no evidence of any other modifications on either the rhAT or the phAT.

Peptide mapping under nonreducing conditions (data not shown) confirmed that the three disulfide linkages occurred between cysteines 8 to 128, 21 to 95, and 247 to 430 as reported previously.[43] The conformation of rhAT was further analyzed by CD spectroscopy (Fig 9). The AT far ultraviolet circular dichroism (CD) spectrum was similar for both proteins and was characterized by two negative bands at 220 to 222 nm and 210 nm and an intense positive band around 193 nm. The two negative minima and a positive maximum were indicative of the presence of both α-helix and β sheet in the protein, which was consistent with the crystal structure data of phAT.[44] The near ultraviolet spectrum of both proteins was also similar with a number of positive and negative peaks between 260 nm and 300 nm (Fig 9B). These data were in excellent agreement with the previously published spectra of AT derived from human and bovine plasma.[45]

We also monitored changes in the CD of AT in the presence of a saturating level of heparin (Fig 9B). In the far ultraviolet spectrum, the addition of heparin produced little change (data not shown), which suggested that secondary structures were not altered. However, the near ultraviolet spectrum showed a dramatic increase in band intensity across the whole region

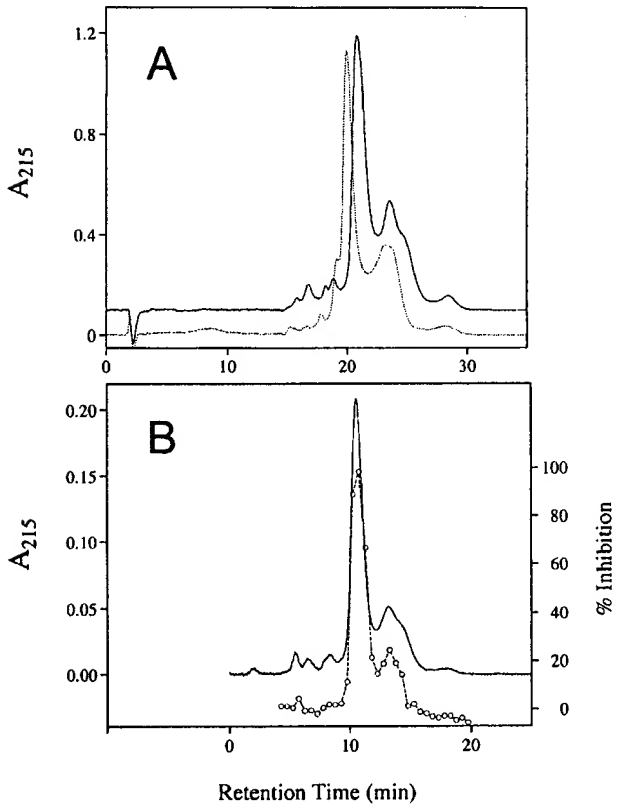


Fig 6. Chromatography of rhAT and phAT on a TSK-Gel Heparin-5PW affinity column. Sample load was 175 μg (A) and 25 μg (B). AT was eluted with a 0- to 3-mol/L NaCl gradient in equilibration buffer with detection at 215 nm. For (A), the dotted line represents phAT and the solid line represents rhAT. For (B), the solid line represents rhAT protein and the circles represent thrombin inhibition activity. The difference in retention times between panels resulted from a 10-minute wash preceding the elution gradient in (A) that was not used in (B).

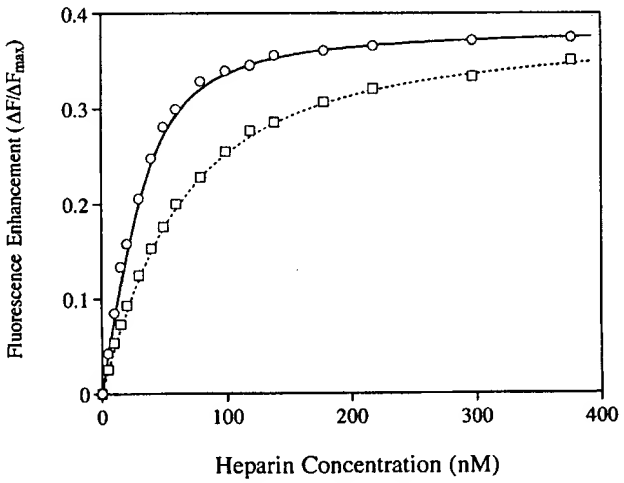


Fig 7. The binding of AT to heparin was analyzed by protein fluorescence enhancement.[41] rhAT (○) and phAT (□) were diluted to 20 nmol/L in 20 mmol/L sodium phosphate, 100 mmol/L NaCl, 100 μmol/L EDTA, 0.1% PEG, and pH 7.4. Heparin was added in 1-μL aliquots, and fluorescence was measured with excitation at 280 nm and emission at 340 nm.

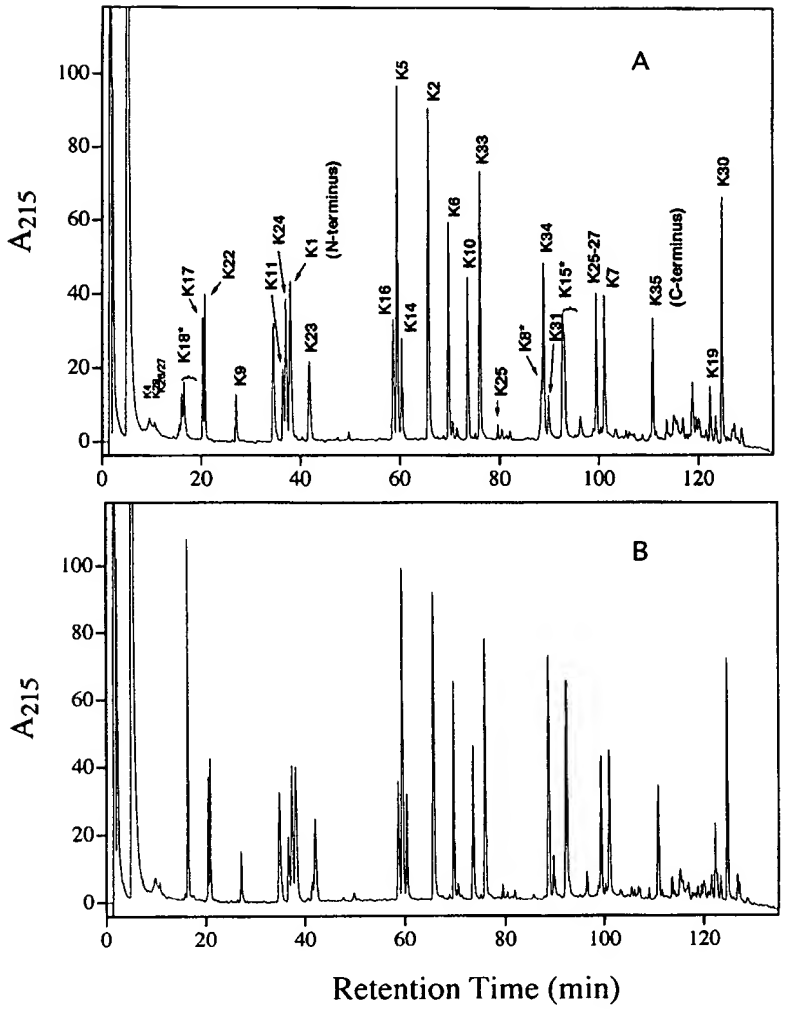


Fig 8. Lys C peptide maps of reduced and alkylated rhAT (A) and phAT (B). The K# designation corresponds to the peptide produced by Lys C digestion as shown in Table 2.

when heparin was added. This marked enhancement in chiral absorption can be attributed mainly to the perturbation of buried and exposed tryptophan residues. This CD result also indicated that for the same protein concentration, the increase in CD intensity was higher for rhAT than for phAT, which again indicated that the heparin affinity of the rhAT was higher than that of phAT.

Carbohydrate analyses. The oligosaccharide structures at all four N-linked glycosylation sites on phAT have been reported to be similar and to contain primarily biantennary disialylated complex oligosaccharides without fucose.[7] The monosaccharide composition obtained for phAT (Table 3) was consistent with such a glycosylation pattern. The monosaccharide composition obtained for rhAT indicated that the glycosylation was significantly different from that of phAT. The key differences were the presence of fucose and GalNAc, a higher level of mannose, a lower level of galactose and sialic acid. No evidence of O-linked glycosylation was observed during LC/MS analysis of the rhAT peptide map, which suggested that the GalNAc is present on N-linked oligosaccharides. The increased level of mannose also suggested the presence of oligomannose structures on the recombinant protein.

The glycosylation heterogeneity of phAT and rhAT was further analyzed by LC/MS analysis of the individual glycopeptides (Fig 10). By using the LC/MS technique, the monosaccharide composition of the oligosaccharide structures present at each site were deduced by subtracting the amino acid mass from the total mass of the glycopeptide. The predominant oligosaccharide observed at each site is presented in Table 4. The glycopeptide containing the Asn 135 site of both phAT and rhAT could not be analyzed in this manner because of its poor retention on the chromatography column. Other LC/MS data indicated that the Asn 135 site of rhAT was greater than 80% glycosylated with the complex oligosaccharide types noted below (data not shown). In humans, AT lacking glycosylation at the Asn 135 site (the β-isoform) was measured at 5% to 15% of the total AT found in plasma.[46,47]

For all three phAT glycopeptides analyzed, the major oligosaccharide species had a mass of 2,206 to 2,207 D. This mass corresponded to a monosaccharide composition of HexNAc4, Hex5,NANA2, which was consistent with a biantennary disialylated structure. This assessment was in agreement with the published data of Franzen et al.[7]

In contrast, the mass spectra obtained for the rhAT glycopeptides are more complex. The mass spectrum obtained for the glycopeptide containing the Asn 96 site (K8) of rhAT (Fig 10A)

Table 2. Lys C Peptide Number, the Residues in Each Peptide With Each Theoretical Mass, and the Masses Observed for rhAT and phAT by LC/MS

Peptide	Residues	Theoretical	Observed Mass	
			rhAT	phAT
K1	1-11	1,232.44	1,232.3	1,232.2
K2	12-28	2,152.61	2,152.5	2,152.6
K3	29-29	146.20	—	—
K4	30-39	1,093.08	1,093.2	1,093.0
K5	40-53	1,698.95	1,698.9	1,698.9
K6	54-70	1,957.14	1,957.2	1,957.2
K7	71-91	2,299.60	2,299.7	2,299.7
K8*	92-107	3,976.18	3,976.6	4,121.9
K9	108-114	838.92	838.7	838.7
K10	115-125	1,340.51	1,340.4	1,340.5
K11	126-133	1,170.46	1,170.2	1,170.3
K12*	134-136	331.38	—	—
K13	137-139	320.35	—	—
K14	140-150	1,219.42	1,219.2	1,219.2
K15*	151-169	3,557.63	3,558.2	4,385.3
K16	170-176	860.03	859.8	859.7
K17	177-188	1,330.43	1,330.3	1,330.3
K18*	189-193	2,693.63	2,693.6	2,839.2
K19	194-222	3,248.78	3,249.1	3,249.9
K20	223-226	502.62	502.3	502.3
K21	227-228	233.28	—	—
K22	229-236	978.08	978.0	977.9
K23	237-241	698.83	698.7	698.4
K24	242-257	1,799.00	1,799.0	1,798.9
K25	258-275	2,209.59	2,209.4	2,209.5
K26	276-287	1,314.62	—	—
K27	288-290	372.43	—	—
K28	291-294	417.51	417.2	417.1
K29	295-297	374.45	—	—
K30	298-332	4,260.98	4,261.5	4,261.0
K31	333-348	1,849.10	1,849.2	1,848.7
K32	349-350	233.28	—	—
K33	351-370	2,202.46	2,202.4	2,202.5
K34	371-403	3,448.85	3,448.8	3,449.1
K35	404-432	3,421.20	3,420.9	3,421.3

*Glycopeptide.

had a major peak at 1,326.5 m/z corresponding to the 3+ charge state of the glycopeptide with a fucosylated biantennary monosialylated oligosaccharide structure. The peak at 995.4 m/z corresponded to the 4+ charge state of the same species. The peak at 1,423.5 m/z corresponded to the fucosylated biantennary disialylated oligosaccharide. Additional minor peaks were observed surrounding the major peaks, which could be accounted for by monosaccharide substitutions. The substitution of an N-glycolyl neuraminic acid (NGNA) for a N-acetyl neuraminic acid (NANA) resulted in a 16-D mass increase, and the substitution of a HexNAc (GalNAc) for Hex (Gal) resulted in a 42-D mass increase. The presence of N-glycolyl neuraminic acid was confirmed by chromatographic analysis. The GalNAc/Gal substitutions accounted for the lower level of galactose and presence of GalNAc in the monosaccharide compositional analysis of rhAT. The spectrum for the phAT Asn 96 glycopeptide (Fig 10D) was less complex with the two major peaks at 1,375 and 1,031.3 m/z corresponding to the 3+ and 4+ charge states of the glycopeptide containing a biantennary disialylated structure.

The mass spectrum of rhAT Asn 155 containing glycopeptide (K15) (Fig 10B), was more heterogeneous than that of the Asn 96 site. This spectrum was dominated by two series of peaks, one centered around the m/z of 1,779.4 (2^+ charge) and one around m/z 1,187 (3^+ charge) and corresponded to a series of masses differing by 162 D representing oligomannose-type oligosaccharides ranging from mannose 3 to mannose 9. The peak with an m/z value of 1,352.1 (3^+ charge) corresponded to the peptide Asn 155 with a hybrid oligosaccharide structure. The peaks with m/z values of 735 and 863 arose from earlier eluting peptides K33 and K34, respectively. In contrast the phAT spectrum for this glycopeptide (Fig 10E) had a single main peak with an m/z value of 1,462.8, again corresponding to a glycopeptide containing a biantennary disialylated oligosaccharide structure. Additionally, treatment of rhAT with endoH to remove oligomannose-type oligosaccharides produced a shift in

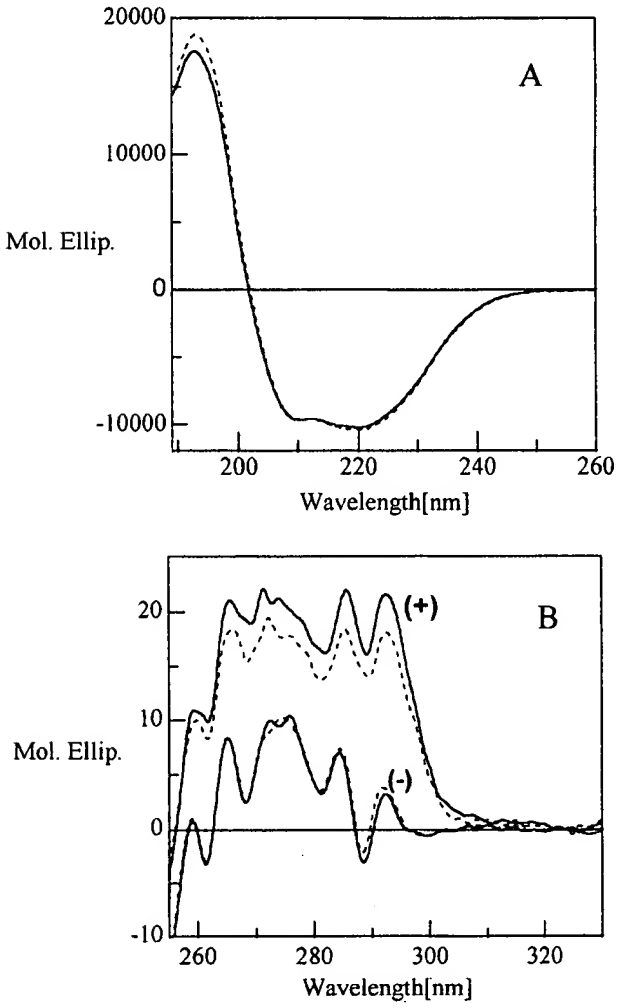


Fig 9. Circular dichroism spectra of rhAT (solid line) and phAT (broken line) in the far ultraviolet (A) and near ultraviolet (B) with (+) and without (−) heparin. The molar ratio between protein and heparin was 1:7. Molar ellipticity is expressed in deg × cm² × dmol $^{-1}$.

Table 3. Monosaccharide Compositions of the Sugars Found on rhAT and phAT

	Moles Sugar/Mole Protein					
	Fucose	GalNAc	GlcNAc	Galactose	Mannose	Sialic Acid
rhAT	2.61 ± 0.15	1.53 ± 0.03	10.78 ± 0.17	4.40 ± 0.03	14.71 ± 0.25	2.84 ± 0.12
phAT	0.00	0.00	13.08 ± 0.01	7.72 ± 0.01	11.03 ± 0.01	5.38 ± 0.28

the peptide map peak containing Asn 155 (K15) (data not shown).

The predominant oligosaccharide at the Asn 192 glycosylation site of rhAT (K18) (Fig 10C) was a fucosylated biantennary monosialylated structure giving an m/z of 899.0 for the 4^+ and 1,348.0 for the 3^+ charge states. The series of peaks around m/z 1,006.6 corresponded to fucosylated biantennary disialylated structures. The heterogeneity observed around these peaks also arose from the monosaccharide substitutions described for the Asn 96 glycopeptide (above). The phAT spectrum (Fig 10F) contained two major peaks at an m/z of 947.3 and 1,420.6 corresponding to the 3^+ and 2^+ charge states of the glycopeptide containing a biantennary disialylated oligosaccharide structure.

FACE analysis of oligosaccharides released from rhAT and phAT confirmed that the predominant oligosaccharide present on the recombinant protein was a monosialylated biantennary fucosylated structure (Fig 11, lane 4). Fucose on the rhAT oligosaccharides made the oligosaccharide slightly larger (slower mobility) than the nonfucoslyated form. The lack of a single sialic acid retarded the mobility of charged oligosaccharides (Fig 11, lane 4) in which the upper major band was identified as the monosialylated and the lower band disialylated. Adding sialic acid to rhAT with sialyltransferase resulted in a FACE gel pattern with shift in intensity from the upper to the lower band (data not shown). The FACE analysis also showed the increased oligosaccharide heterogeneity present on rhAT compared with both phAT and goat plasma AT although the method did not clearly resolve the oligomannose structures in the rhAT sample. That the FACE profile of goat AT was similar to that of phAT suggested that the increased fucosylation and heterogeneity, as well as the presence of oligomannose structures, was a result of expression in the mammary gland and did not arise because of goat/human glycosylation differences.

DISCUSSION

Production of rhAT in the mammary gland of transgenic goats has provided a means for high level (>1 g/L) expression of this therapeutic protein. This report provides the first detailed structural and functional analysis of a human therapeutic protein produced in the milk of a transgenic animal to be used in human clinical studies. In fact, rhAT was successfully evaluated in phase I and phase II human clinical studies designed to test the ability of rhAT to maintain hemostasis during coronary artery bypass graft surgery.

The rhAT isolated from the milk of transgenic goats was indistinguishable from phAT with the exception of its heparin-binding affinity and the nature of its glycosylation. The rhAT

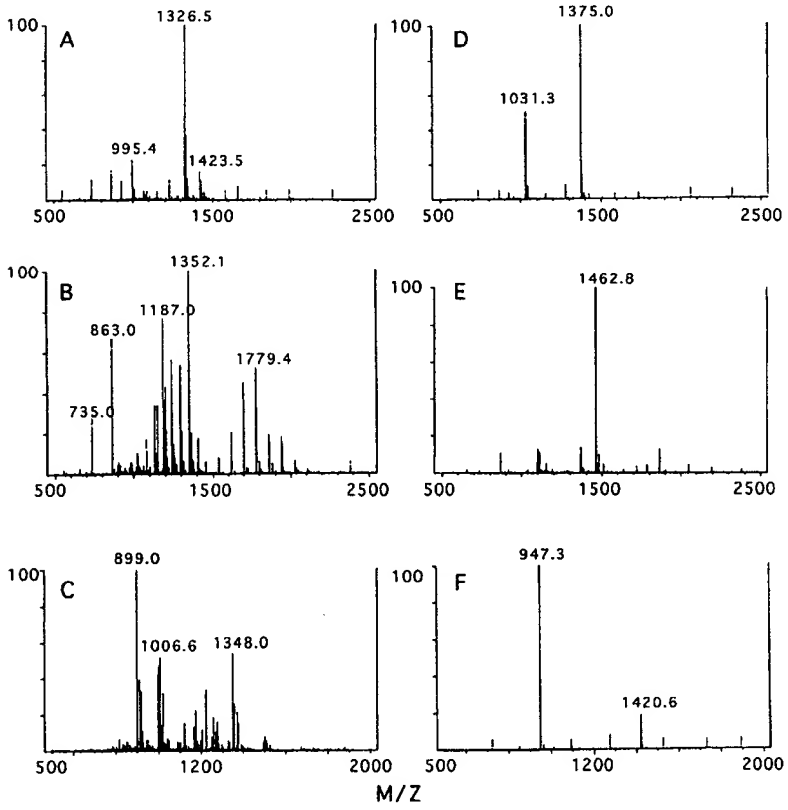


Fig 10. The mass spectrum obtained for the glycopeptides containing Asn 96, Asn 155, and Asn 192 for rhAT (A, B, and C) and phAT (D, E, and F), respectively.

Table 4. Major Oligosaccharide Structures of Glycosylation Sites Deduced From LC/MS Data of Glycopeptides

Site	Peptide Mass	Observed Mass	Observed CHO Mass	Theoretical CHO Mass	Composition
rhAT					
Asn 96	1,915.3	3,976.5	2,061.2	2,060.9	HexNAc4,Hex5, Fuc1,NANA1
Asn 155	2,178.4	3,558.0	1,379.6	1,379.2	HexNAc2,Hex6
Asn 192	632.7	2,694.0	2,061.3	2,060.9	HexNAc4,Hex5, Fuc1,NANA1
phAT					
Asn 96	1,915.3	4,122.0	2,206.7	2,206.0	HexNAc4,Hex5, NANA2
Asn 155	2,178.4	4,385.4	2,207.0	2,206.0	HexNAc4,Hex5, NANA2
Asn 192	632.7	2,838.9	2,206.2	2,206.0	HexNAc4,Hex5, NANA2

and phAT exhibited equivalent activity in in vitro thrombin and factor Xa inhibition assays and were structurally comparable in RP-HPLC binding characteristics, LC/MS peptide mapping profiles (except for the glycopeptides) and near and far ultraviolet circular dichroism spectra. The major differences in glycosylation noted were that the rhAT was less sialylated, more fucosylated, and contained GalNAc for Gal substitutions and some NGNA as well as NANA as terminal sugars when compared with phAT.

These differences in glycosylation between the rhAT and phAT are presumed responsible for the difference in heparin binding. Previous reports by Zettlmeissl et al[48] described site mutation experiments on individual glycosylation sites of rhAT produced in Chinese hamster ovary (CHO) cells, which resulted in higher affinity for heparin when compared with phAT. A current report,[49] using similar techniques, corroborated the above study and further showed that the presence of glycosylation on the Asn 155 site was responsible for generating the lower affinity glycoform of the fully glycosylated AT made in CHO cells. The nature of the oligosaccharides present was not determined. In another study, Garone et al[50] reported that fucosylation at the Asn 155 site reduced the heparin affinity of a variant of rhAT made in baby hamster kidney cells in which Asn 135 was mutated to Gln 135. Neither the phAT nor the rhAT examined here had fucosylated oligosaccharides at the Asn 155 site. The main difference observed between phAT and rhAT at Asn 155 was in the nature of the glycosylation: phAT had a charged oligosaccharide and rhAT had a noncharged oligosaccharide. Because of the different expression systems used and differences in glycosylation at sites other than the Asn 155 site, it is difficult to directly compare the results we have obtained with these other studies. However, it is apparent that glycosylation differences at the Asn 155 site are important in determining the overall heparin affinity of AT.

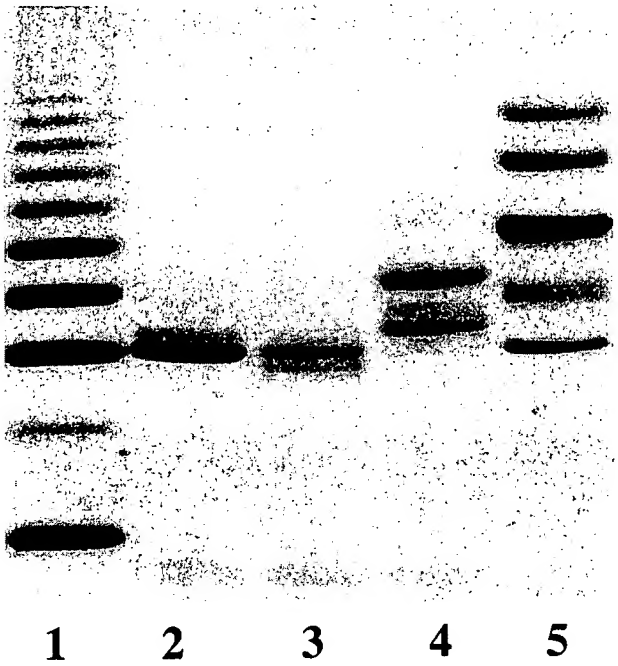


Fig 11. FACE analysis of phAT (lane 2), goat plasma AT (lane 3), and rhAT (lane 4) N-glycanase released oligosaccharides. Lane 1 contains dextran polymers of varying length beginning with a trimer at the bottom of the gel and increasing by one sugar for each band to the top. Lane 5 contains a series of standard oligosaccharides (Dionex Corp) from bottom to top: disialylated biantennary, trisialylated triantennary, asialo biantennary, asialo triantennary, and asialotetraantennary complex oligosaccharides.

The data presented above indicated that both the α and β isoforms of rhAT bound more tightly to heparin than the α and β isoforms of phAT. Because all of the sites of the α form are fully glycosylated, the increase in heparin affinity observed must have been caused by the nature of the glycosylation present not the degree of site occupancy. As noted above, rhAT exhibited an overall lower level of sialylation than phAT and a very different glycoform distribution at the Asn 155 site. Initial studies suggested that removal of sialic acid from phAT resulted in a higher affinity for heparin (data not shown). The prevention of glycosylation on the individual sites increased heparin affinity[48,49] purportedly by reducing steric hindrance to heparin binding. Alternatively, the prevention of glycosylation by site mutation also prevented potential sialylation, which could exert a charge repulsion effect with negatively charged heparin sulfate. Whether the absence of sialic acid on specific glycosylation sites would impart any enhancement of heparin binding will require further study.

The fact that transgenically produced rhAT had a higher heparin affinity may have relevance to its clinical use. The importance of an increased affinity of AT for heparin in vivo may lie in the fact that heparin and heparin-like glycosaminoglycans are receptors for AT and thrombin and that these glycosaminoglycans are found in the vascular endothelium and extravascular spaces, not circulating in the blood stream. AT is activated by binding to heparan sulfate proteoglycans on endothelial cells,[17,21,51,52] and clearance of AT from blood occurs by redistribution into the extravascular space and binding to the endothelial surfaces.[24,51,53]

The control of thrombin activity locally may be the initial step in preventing disseminated intravascular coagulation (DIC) by controlling the occurrence of coagulatable sites in blood vessels and organs.[21] Thrombin was implicated in stimulating the vascular endothelium to synthesize prostacyclin,[54] tissue plasminogen activator,[55] and tissue plasminogen activator inhibitor[56] besides its activation of coagulation. AT was shown to

inhibit thrombin-induced prostacyclin induction in HUVEC cells.[57] Thrombin was also found to enhance the adhesion of neutrophil leukocytes to endothelial cells.[58,59]

A recent study showed that AT at high dose, without added heparin, was an effective and safe therapy for disseminated intravascular coagulation with organ failure in four children.[60] Thus, enhancing the ability of AT to inhibit thrombin locally may provide a better way to control not just coagulation but other thrombin-induced events. rhAT, with its higher affinity for heparin, could provide more AT at sites of insult or inflammation to help control DIC and other events stimulated by the presence of thrombin. Studies to ascertain differences in the biodistribution and efficacy because of increased heparin affinity are in progress.

ACKNOWLEDGMENT

We thank Paul DiTullio, currently of Midas Biologicals Inc, (Grafton, MA) for his extensive efforts in making this program possible. We also thank Karen Albee, Karen Lee, and Robert Segalla of the Structural Protein Chemistry Group for providing expert analytical support.

REFERENCES

1. Travis J, Salvesen GS: Human plasma proteinase inhibitors. Annu Rev Biochem 52:655, 1983

2. Menache D: Antithrombin III: Introduction. Semin Hematol 28:1, 1991

3. Menache D, Grossman B, Jackson C: Antithrombin III: Physiology, deficiency, and replacement therapy. Transfusion 32:580, 1992

4. Lahiri B, Bagdasarian A, Mitchell B, Talamo RC, Colman RW, Rosenberg RD: Antithrombin-Heparin Cofactor: An inhibitor of plasma kallikrein. Arch Biochem Biophys 175:737, 1976

5. Rosenberg RD, Bauer KA, Marcum JA: Antithrombin III, The heparin antithrombin system. Rev Hematol 2:351, 1986

6. Murano G, Williams L, Andersson M: Some properties of antithrombin III and its concentration in human plasma. Thromb Res 18:259, 1980

7. Franzen LE, Svensson S, Larm O: Structural studies on the carbohydrate portion of human antithrombin III. J Biol Chem 255:5090, 1980

8. Peterson T, Dudek-Wojcicchowska G, Sottrup-Jensen L, Magnusson S: Primary structure of antithrombin III (Heparin Cofactor). Partial homology between α_1 antitrypsin and antithrombin-III, in Collen DW, Wiman B, Verstraete M (eds): The Physiological Inhibitions of Blood Coagulation and Fibrinolysis. Amsterdam, The Netherlands, Elsevier/North-Holland Biomedical Press, 1979, p 43

9. Pike R, Potempa J, Skinner R, Fitton H, McGraw W, Travis J, Owen M, Jin L, Carrell R: Heparin-dependent modification of the reactive center arginine of antithrombin and consequent increase in heparin binding affinity. J Biol Chem 272:19652, 1997

10. Ersdal-Badju E, Lu A, Zuo Y, Picard V, Bock S: Identification of the antithrombin III heparin binding site. J Biol Chem 272:19393, 1997

11. Rosenberg RD: Role of heparin and heparinlike molecules in thrombosis and atherosclerosis. Fed Proc 44:404, 1985

12. Bjork I, Danielsson A: Antithrombin and related inhibitors of coagulation proteinases, in Barrett AJ, Salvesen GS (eds): Proteinase Inhibitors, vol 17. Amsterdam, The Netherlands, Elsevier Science Publishers (Biomedical Division), 1986, p 489

13. Olson ST, Bjork I, Sheffer R, Craig PA, Shore JD, Choay J: Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement. J Biol Chem 267:12528, 1992

14. Choay J, Lormeau JC, Petitou M, Sinay P, Fareed J: Structural studies on a biologically active hexasaccharide obtained from heparin. Ann NY Acad Sci 370:644, 1981

15. Choay J, Petitou M, Lormeau J-C, Sinay P, Casu B, Gatti G: Structure activity relationship in heparin: A synthetic pentasaccharide sequence with high affinity for antithrombin III and eliciting high anti factor Xa activity. Biochem Biophys Res Commun 116:492, 1983

16. Olson ST, Bjork I: Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects. J Biol Chem 266:6353, 1991

17. Bauer KA, Rosenberg RD: Role of antithrombin III as a regulator of in vivo coagulation. Semin Hematol 28:10, 1991

18. Carrell R, Skinner R, Jin L, Abrahams J: Structural mobility of antithrombin and its modulation by heparin. Thromb Haemost 78:516, 1997

19. Hashimoto K, Yamagishi M, Sasaki T, Nakano M, Kurosawa H: Heparin and Antithrombin III levels during Cardiopulmonary bypass: Correlation with subclinical plasma coagulation. Ann Thorac Surg 58:799, 1994

20. Cloyd GM, D'Ambra MN, Akins CW: Diminished anticoagulant response to heparin in patients undergoing coronary artery bypass grafting. J Thorac Cardiovasc Surg 94:535, 1987

21. Rosenberg RD: Biochemistry of heparin antithrombin interactions, and the physiologic role of this natural anticoagulant mechanism. Am J Med 87:2S, 1989 (suppl)

22. Mertens G, Cassiman J-J, Van den Berghe H, Vermylen J, David G: Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. J Biol Chem 267:20435, 1992

23. Akai T, Kaji T, Hayakawa Y, Hayashi T, Sakuragawa N: Antithrombin modulates the effect of thrombin in the metabolism of glycosaminoglycans in cultured endothelial cells. Thrombosis Res 62:707, 1991

24. de-Agostini AI, Watkins SC, Slayter HS, Youssoufian H, Rosenberg RD: Localization of anticoagulantly active heparan sulfate proteoglycans in vascular endothelium: Antithrombin binding on cultured endothelial cells and perfused rat aorta. J Cell Biol 111:1293, 1990

25. Ebert KM, Selgrath JP, DiTullio P, Denman J, Smith TE, Memon MA, Schindler JE, Monastersky GM, Vitale JA, Gordon C: Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: Generation of transgenic goats and analysis of expression. Bio/Technology 9:835, 1991

26. Denman J, Hayes M, O'Day C, Edmunds T, Bartlett C, Hirani S, Ebert KM, Gordon K, McPherson JM: Transgenic expression of a variant of human tissue-type plasminogen activator in goat milk: Purification and characterization of the recombinant enzyme. Bio/Technology 9:839, 1991

27. Ebert KM, DiTullio P, Barry CA, Schindler JE, Ayres SL, Smith TE, Pellerin LJ, Meade HM, Denman J, Roberts B: Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. Bio/Technology 12:699, 1994

28. Varki A: Biological roles of oligosaccharides: All of the theories are correct. Glycobiology 3:97, 1993

29. Roberts B, DiTullio P, Vitale J, Hehir K, Gordon K: Cloning of the goat-casein encoding gene and expression in transgenic mice. Gene 121:255, 1992

30. DiTullio P, Cheng SH, Marshal J, Gregory RJ, Ebert KM, Meade HM, Smith AE: Production of cystic fibrosis transmembrane regulator in the milk of transgenic mice. Bio/Technology 10:74, 1992

31. Broker M, Ragg H, Karges HE: Expression of human antithrombin III in Saccharomyces cerevisiae and Schizosaccharomyces pombe. Biochim Biophys Acta 908:203, 1987

32. Gould SJ, Subramani S, Scheffler IE: Use of the DNA polymerase chain reaction for homology probing: Isolation of partial cDNA or genomic clones encoding the iron-sulfur protein succinate dehydrogenase from several species. Proc Natl Acad Sci USA 86:1934, 1989

33. Abildgaard U, Lie M, Odegard OR: Antithrombin (heparin cofactor) assay with "new" chromogenic substrates (S-2238 and Chromozym TH). Thromb Res 11:549, 1977

34. Abildgaard U: Methodological considerations on antithrombin determination. Acta Chir Scand Suppl 509:97, 1982

35. Cole ES, Nichols EH, Lauziere K, Edmunds T, McPherson JM: Characterization of the microheterogeneity of recombinant primate prolactin: Implications for posttranslational modifications of the hormone in vivo. Endocrinology 129:2639, 1991

36. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

37. Morrissey JH: Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307, 1981

38. Burnette WN: "Western Blotting." Electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 170:195, 1981

39. Hardy MR, Townsend RR, Lee YC: Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. Anal Biochem 170:54, 1988

40. Powell LD, Hart GW: Quantitation of picomole levels of N-acetyl- and N-glycolylneuraminic acids by a HPLC-adaptation of the thiobarbituric acid assay. Anal Biochem 157:179, 1986

41. Fan B, Crews BC, Turko IV, Choay J, Zettlmeissl G, Gettins P: Heterogeneity of recombinant human antithrombin III expressed in baby hamster kidney cells. Effect of glycosylation differences on heparin binding and structure. J Biol Chem 268:17588, 1993

42. Knobloch J, Shaklee P: Absolute molecular weight distribution of low-molecular weight heparins by size-exclusion chromatography with multiangle laser light scattering detection. Anal Biochem 245:231, 1997

43. Carrell RW, Stein PE, Fermi G, Wardell MR: Biological implications of a 3A structure of dimeric antithrombin. Structure 2:257, 1994

44. Schreuder HA, de-Boer B, Dijkema R, Mulders J, Theunissen HJ, Grootenhuis PD, Hol WG: The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. Nat Struct Biol 1:48, 1994

45. Nordenman B, Nystrom C, Bjork I: The size and shape of human and bovine antithrombin III. Eur J Biochem 78:195, 1977

46. Frebelius S, Isaksson S, Swedenborg J: Thrombin inhibition by antithrombin III on the subendothelium is explained by the isoform AT beta. Arterioscler Thromb Vasc Biol 16:1292, 1996

47. Peterson CB, Blackburn MN: Isolation and characterization of an antithrombin III variant with reduced carbohydrate content and enhanced heparin binding. J Biol Chem 260:610, 1985

48. Zettlmeissl G, Conradt HS, Nimtz M, Haigwood N, Paques EP: Influence of glycosylation on the functional properties of human therapeutic plasma proteins. GBF Monogr Ser 15:259, 1991

49. Olson S, Frances-Chmura A, Swanson R, Bjork I, Zettlmeissl G: Effect of individual carbohydrate chains of recombinant antithrombin on heparin affinity and on the generation of glycoforms differing in heparin affinity. Arch Biochem Biophys 341:212, 1997

50. Garone L, Edmunds T, Hanson E, Bernasconi R, Huntington JA, Meagher JL, Fan B, Gettins PGW: Antithrombin-heparin affinity reduced by fucosylation of carbohydrate at asparagine 155. Biochemistry 35:8881, 1996

51. Hatton MWC, Moar SL, Richardson M: On the interaction of rabbit antithrombin III with the luminal surface of the normal and de-endothelialized rabbit thoracic aorta in vitro. Blood 67:878, 1986

52. Hatton MWC, Moar SL, Richardson M: Evidence that rabbit ^{125}I-antithrombin III binds to proteoheparan sulphate at the subendothelium of the rabbit aorta *in vitro*. Blood Vessels 25:12, 1988

53. Pizzo SV: Serpin receptor 1: A hepatic receptor that mediates the clearance of antithrombin III-proteinase complexes. Am J Med 87:10S, 1989 (suppl)

54. Weksler BB, Ley CW, Jaffe EA: Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and theionophore A23187. J Clin Invest 62:923, 1978

55. Levin EG, Loskutoff PJ: Cultured bovine endothelial cells produce both urokinase and tissue-type plasminogen activators. J Cell Biol 94:631, 1982

56. Gelehter TJ, Sznycer-Laszuk R: Thrombin induction of plasminogen activator-inhibitor in cultured human endothelial cells. J Clin Invest 77:165, 1986

57. Kaji T, Itoh F, Hayakawa Y, Oguma Y, Sakurgawa N: Interaction of thrombin with heparin cofactor II and antithrombin III on prostacyclin production by cultured endothelial cells. Thromb Res 56:99, 1989

58. Toothill VJ, Van Mourik JS, Niewenhuis hK, Metzelaar MJ, Pearson JD: Characterization of the enhanced adhesion of neutrophil leukocytes to thrombin-stimulated endothelial cells. J Immunol 145: 283, 1990

59. Moser R, Groscurth P, Fehr J: Promotion of transendothelial neutrophil passage by human thrombin. J Cell Science 96:737, 1990

60. Fuse S, Tomita H, Yoshida M, Hori T, Igarashi C, Fujita S: High dose of intravenous antithrombin III without heparin in the treatment of disseminated intravascular coagulation and organ failure in four children. Am J Hematol 53:18, 1996

G

Recombinant Human AT III BB-IND 6881 Activites
(Heparin Resistance Indication)

Date	Serial No.	Content
November 5, 1993	--	Initial communication with FDA regarding GTC rhAT III program.
April 12, 1994	--	Request for pre-IND meeting.
May 5, 1994	--	Telecon from FDA conveying comments on the Nov. 5, 1993 correspondence.
July 22, 1994	--	Responses to FDA's May 5 comments
August 9, 1994	--	Initial pre-IND type meeting
May 17, 1996	--	Pre-IND meeting to follow-up on issues
October 15, 1996	000	Original IND application
Nov. 13, 1996	--	Telecon with FDA wherein FDA requested modifications of the study protocols prior to being authorized to proceed with the study.
Nov. 14, 1996	--	Committed to make the protocol modifications referred to above and, as such, were authorized to proceed with the study.
December 12, 1996	--	Letter from FDA requesting confirmation of protocol changes and specifications for finished product.
January 27, 1997	002	Protocol Amendment, investigator documentation, CRF, consent form, IRB approval, and final report for the Phase I study
April 3, 1997	004	Protocol Amendment with changes to the general investigational plan and contains a final report
April 15, 1997	005	Notification of increased dosage together with protocol amendment
June 5, 1997	006	Response to FDA letter dated Dec. 12, 1996
June 27, 1997	007	Request for End-of-Phase II meeting to discuss Phase III study protocols (includes proposed protocols)
July 29, 1997	--	End-of-Phase II meeting
Sept. 12, 1997	008	Request for conference call with FDA to discuss Phase III clinical development plan.
Sept. 25, 1997	--	Teleconference with FDA
Oct. 15, 1997	009	Submission of revised Phase III study protocols for FDA review and comment.
Nov. 18, 1997	010	Documentation for Nov. 24, 1997 teleconference with FDA to discuss the clinical development plan
Nov. 24, 1997	--	Teleconference with FDA
Dec. 18, 1997	011	First Annual Report
Jan. 9, 1998	--	Letter from FDA regarding the clinical development plan for rhATIII
April 8, 1998	012	Responses to FDA's Jan. 9 letter
August 13, 1998	013	Information amendment containing final reports for the Phase I-II clinical trial. Notifies FDA regarding initiating the Phase III trials.
Aug. 14, 1998	--	Letter from FDA with comments on the clinical

Recombinant Human AT III BB-IND 6881 Activites
(Heparin Resistance Indication)

		development plan
Oct. 9, 1998	015	Response to FDA's Aug. 14 letter with amendments to the Phase III protocols
Nov. 16, 1998	017	Request for meeting to discuss a "harmonized" license application as opposed to a BLA
Dec. 14, 1998	--	Letter from FDA regarding the clinical development plan
Jan. 15, 1999	018	Annual Report
Jan. 21, 1999	019	In lieu of meeting, provided FDA with outline of "harmonized" application with request for FDA feedback
Jan. 25, 1999	020	Protocol amendment based on conversation with FDA
April 1, 1999	022	Comparability protocol for heat-treated product
April 16, 1999	024	Information Amendment: GTC article published in Nature Biotechnology
April 21, 1999	025	Response to FDA's Dec. 18 letter and additional investigator documentation
June 18, 1999	029	Protocol amendment for the Phase III study
Aug. 17, 1999	033	Further clarifications to the April 21, 1999 responses
Nov. 18, 1999	034	Notifies FDA that Company believes it has addressed all of FDA's comments/recommendations regarding Phase III studies and requests written confirmation.
Jan. 12, 2000	035	Annual Report
March 2, 2000	--	Letter from FDA in response to Nov. 18, 1999 request
June 28, 2000	037	Provides data from analyses of heat-treated rhAT III and provides protocol for Phase I PK study
Aug. 7, 2000		Letter from FDA about antibody analysis
Sept. 7, 2000	038	Proposed new protocol for a Phase I study
Oct. 3, 2000	040	Provided responses to FDA's March 2, 2000 letter
Nov. 9, 2000	041	Request for meeting
Dec. 20, 2000	042	Meeting briefing package
Jan. 16, 2001	--	Meeting with FDA
Jan. 18, 2001	043	Annual Report
Jan. 31, 2001		FDA's minutes of Jan. 16 meeting
Dec. 13, 2001	053	Comparability data and human PK data
Jan. 18, 2002	054	Final reports submitted notifying FDA that the Company decided not to pursue a license application at this time
Feb. 21, 2002	057	Annual Report
Nov. 6, 2002	059	Request for meeting to discuss clinical development plan for the HD indication
Nov. 20, 2002	060	Briefing Package for Dec. 17, 2002 teleconference to discuss clinical plan for HD indication.
Dec. 23, 2002	061	Information Amendment: Pharm./Tox. Repro-toxicology study report

Recombinant Human AT III BB-IND 6881 Activites
(Heparin Resistance Indication)

May 3, 2005		Letter from FDA regarding annual report
June 6, 2005	062	Inactivate IND
June 21, 2005		Letter from FDA inactivating IND 6881

Recombinant Human AT III BB-IND 10941 Activites
(Hereditary Deficiency Indication)

Serial #	Correspondence	Date	To:	From:	Content
	Letter	10-Jan-03	R. Scotland	K. Needleman	12-Dec-07 telephone memo
000	Letter, 2 Vols (X 3)	28-Feb-03	G. Jones	R. Scotland	IND
	Fax	10-Mar-03	Ilan Irony	R. Scotland	Protocol synopsis, IB pages on prev trials
	Letter	12-Mar-03	R. Scotland	K. Needleman	IND submission received
	Fax	12-Mar-03	Ilan Irony	R. Scotland	Additional info Dosing regiment/AT levels
	Letter, 2 Vols (X 6)	14-Mar-03	K. Needleman	R. Scotland	Additional IND copies
	Fax	14-Mar-03	Ilan Irony	R. Scotland	Communication regarding amending protocol
	Fax	1-Apr-03	Ilan Irony	R. Scotland	Informational minutes and telecon
	Fax	1-Apr-03	Ilan Irony	R. Scotland	Confirming that IND went into effect
	Fax	3-Apr-03	R. Wagner	R. Scotland	Informational - pre-IND package
	Fax	4-Apr-03	R. Wagner	R. Scotland	Stability Data
001	Letter, 1 Vol	1-Jul-03	Ilan Irony	R. Scotland	Protocol Amendment 3, New Investigator
	Letter	2-Jun-03	R. Scotland	G. Jones	Protocol Revision letter
	Letter	30-Jul-03	H. Balick	R. Scotland	Informational correspondence, minutes
002	Letter, 1 Vol	4-Sep-03	J. Anderson	R. Scotland	Information Amendment, immunosurveillance
005	Letter, 1 Vol	7-Oct-03	B. Goulding	R. Scotland	Response to Request for Information
006	Letter, append	8-Oct-03	B. Goulding	R. Scotland	IND Safety Report
008	Letter, 2 Vols	8-May-04	B. Goulding	R. Scotland	PK - Study information
009	Letter, 3 Vols	8-May-04	B. Goulding	R. Scotland	Clinical Study information
010	Letter	8-May-04	B. Goulding	R. Scotland	End of Phase II Meeting Request
	Letter	19-May-04	R. Scotland	S. Nedjar	30-Apr-04 Meeting minutes
011	Letter	13-Feb-04	R. Scotland	S. Nedjar	Withdrawal of Meeting Request
012	Letter	3-Mar-04	R. Scotland	S. Nedjar	End of Phase II Meeting Request
013	Letter, 1 Vol (X 10)	19-Mar-04	B. Goulding	R. Scotland	Pre-meeting Information Package
014	Letter, append	8-May-04	B. Goulding	R. Scotland	Protocol Amendment
015	Letter	20-Aug-04	B. Goulding	R. Scotland	LOA - investigator-sponsored IND
016	Letter, 1 Vol	3-Sep-04	B. Goulding	R. Scotland	Information Amendment - CMC
017	Letter, 1 Vol	3-Sep-04	B. Goulding	R. Scotland	Protocol Amendment
018	Letter, 1 Vol	20-Sep-04	B. Goulding	R. Scotland	FU - PK Trial
019		20-Sep-04	B. Goulding	R. Scotland	Protocol Amendment
	Fax- Informal communication	28-Sep-04	B. Goulding	R. Scotland	Clinical info Phase I
021	Letter, 1 Vol	25-Feb-05	B. Goulding	R. Scotland	Protocol Amendment & SAP
	Fax	8-Apr-05	J. Anderson	R. Scotland	Modified protocol pages
022	Letter, 1 Vol	8-Apr-05	B. Goulding	R. Scotland	Final Protocols & SAP
023	Letter, 1 Vol	4-Nov-05	B. Goulding	R. Scotland	Revised Protocol and IB

Recombinant Human AT III BB-IND 10941 Activites
(Hereditary Deficiency Indication)

Serial #	Correspondence	Date	To:	From:	Content
024	Letter, 1 Vol	30-Jun-06	B. Goulding	R. Scotland	IND Annual Report
025	Letter, 1 Vol	22-Sep-06	B. Goulding	R. Scotland	Protocol Amendment 2
026	Letter	29-Mar-07	B. Goulding	R. Scotland	Request to Submit Rolling BLA
027	Letter, 1 Vol	4-Apr-07	B. Goulding	R. Scotland	New Investigator
028	Letter, 1 Vol	26-Apr-07	B. Goulding	R. Scotland	IND Annual Report
029	Letter, 1 Vol	4-Jun-07	B. Goulding	R. Scotland	New Investigator
030	Letter, 1 Vol	8-Jun-07	B. Goulding	R. Scotland	Initial IND Safety Report
031	Letter, 1 Vol	21-Jun-07	B. Goulding	R. Scotland	Follow-up IND Safety Report
032	Letter, 1 Vol	3-Jul-07	B. Goulding	R. Scotland	Follow-up # 2 IND Safety Report
033	Letter	19-Jul-07	B. Goulding	R. Scotland	Request Fast Track Designation
034	Fax	30-Jul-07	B. Goulding	R. Scotland	Request Type B Meeting (pre-BLA)
035	Letter, 1 Vol	9-Aug-07	B. Goulding	R. Scotland	Pre-BLA Information Package
	Fax	29-Aug-07	R. Scotland	P. Rana	Meeting Schedule
	Letter	30-Aug-07	R. Scotland	B. Goulding	Fast Track acceptance letter
036		21-Sep-07	B. Goulding	R. Scotland	Response to Requests - Immunoassays
	Fax	5-Oct-07	R. Scotland	P. Rana	Response to questions of pre-BLA package
	Letter, CD	7-Nov-07	M. Fauntleroy	R. Scotland	Demo CD BLA
037	Letter, 1 Vol	15-Nov-07	B. Goulding	R. Scotland	IND Safety Report
	Phone	16-Nov-07	Dr. Startzman	R. Scotland	Follow-up to Telecon on additional information
038	Letter, 1 Vol	12-Dec-07	B. Goulding	R. Scotland	Follow-up # 1IND Safety Report
039	Letter, 1 Vol	24-Jan-08	B. Goulding	R. Scotland	New Investigator
040	Letter, 1 Vol	19-Feb-08	B. Goulding	R. Scotland	Follow-up # 2 IND Safety Report

Recombinant Human AT III BLA-125284 Activites

Seruence #	Correspondence	Date	To:	From:	Content
	1 DVD	7-Nov-07	M.Fauntleroy	R. Scotland	Letter and Pre-BLA Demo CD
		31-Jan-08	FDA	R. Scotland	FDA User Fee Account & Confirmation
0000	1 DVD	31-Jan-08	B. Golding	R. Scotland	eCTD Part 1 Original Biologic License Application
	Letter	29-Feb-08	R. Scotland	P. Rana	Receipt of BLA
0001	1 DVD	14-Mar-08	B. Golding	R. Scotland	Amendment. Response to 13-Mar-08 teleconference.
	Letter	27-Mar-08	FDA	R. Scotland	Initial Establishment Registration - Form FDA 2656
					GMP Inspection
0002	1 DVD	11-Apr-08	B. Goulding	R. Scotland	Investigator List/efficacy tables
	e-mail	May-08	Chiang	R. Scotland	FDA Correspondence re: Inspection
	Letter	9-May-08	Drews/Syin	R. Scotland	Responses to Observations to Apr. 10-18 pre approval Inspection
	Correspondence	10-Jun-08	Greg Liposky	R. Scotland	Telecon with FDA
0003	Letter	6-Aug-08	B. Golding	R. Scotland	Part 2 Cover Letter of Rolling Biologic License Application
	Letter/FDA Form 356h/DVD	6-Aug-08	B. Golding	R. Scotland	Letter/FDA Form356h with DVD (1 of 1) - Part 2 Rolling BLA
	Correspondence	7-Aug-08	B. Golding	R. Scotland	Correspondence: Submission of Part 2 Rolling BLA
	Correspondence	8-Aug-08	P.Rana	R. Scotland	Correspondence/Cover Letter 1.2 Part 2 Rolling BLA
	Correspondence	22-Aug-08	R. Scotland	P. Rana	Letter Re: Action Due Date February 07, 2009
	Fax	22-Aug-08	R. Scotland	J.Durham/ R.Rana	Fax Re: Action Due Date
	Fax	17-Sep-08	R. Scotland	P. Rana	Fax: Request for Information
	Telephone Message	23-Sep-08	R. Scotland	R.Drews	Tel. Message/Contact Report: Sending another Information Request
	Correspondence	23-Sep-08	R. Drews	R. Scotland	Confirmation of Information Request
	Fax	23-Sep-08	R. Scotland	P. Rana	Request for Information
	Fax	29-Sep-08	R.Scotland	P. Rana	Fax Re: Request for Information
	Correspondence	29-Sep-08	R. Drews	R.Scotland	Correspondence Re: Call from R. Drews
0005	Letter	1-Oct-08	B. Golding	R.Scotland	Letter re: Seq. 0005 Response to FDA Fax 17SEP08
	Fax from FDA	2-Oct-08	R. Scotland	FDA	FDA Filing Letter
0006	Letter	8-Oct-08	B. Golding	R.Scotland	Letter re: Seq. 0006 Response to FDA Fax 23SEP08

Recombinant Human AT III BLA-125284 Activites

Sequence #	Correspondence	Date	To:	From:	Content
Seq - 0007	Letter/356h/ Responses/CD	14-Oct-08	B. Golding	R. Scotland	14Oct08 Letter - Seq. 0007
Seq - 0008	Letter/356h/ Responses/CD	28-Oct-08	B. Golding	R. Scotland	28Oct08 Letter - Seq. 0008
Seq - 0009	Letter/356h/ Responses/CD	5-Nov-08	B. Golding	R. Scotland	05Nov08 Letter - Seq. 0009
	Correspondence	14-Nov-08	Chiang	R. Scotland	Correspondence\14 Nov 08 Email correspondence.pdf
Seq 0010	Letter/356h/CD	14-Nov-08	B. Golding	R. Scotland	14Nov08 - Seq. 0010
Seq - 0011	Letter/356h/CD	18-Nov-08	B. Golding	R. Scotland	18Nov08 Letter - Seq. 0011
Seq - 0012	Letter/356h/CD	19-Nov-08	B. Golding	R. Scotland	19Nov08 Letter - Seq. 0012
	Correspondence	24-Nov-08	N. Jain	R. Scotland	24Nov08 Email Correspondence (2 Articles)
	Correspondence	24-Nov-08	N. Jain	R. Scotland	24Nov08 Email Correspondence (2)
	Correspondence	1-Dec-08	N. Jain	R. Scotland	Email Correspondence 01Dec08
	Letter - Advisory Briefing Package/DVD	4-Dec-08	D.Jehn	R. Scotland	04Dec08 Letter - Briefing Package
	Letter - Sample Request	8-Dec-08	S. Dougherty	R. Scotland	08Dec08 Letter - Sample Request
Seq 0013	Letter/356h/CD	16-Dec-08	Dr. Golding	R. Scotland	Seq 0013-DS-DP Specifications
Seq - 0014	Letter/356h/CD	16-Dec-08	Dr. Golding	R. Scotland	Seq. 0014-Patient Ultrasounds
	Correspondence	17-Dec-08	R. Scotland	P. Rani	Request for DS-DP Specification Information
	Correspondence	17-Dec-08	R. Scotland	Heather Murray	Advisory Committee Meeting Instructions
Seq - 0015	Letter/356h/CD	24-Dec-09	Dr. Golding	R. Scotland	Seq. 0015-Stability Data and Protocols
Seq - 0016	Letter/356h/CD	24-Dec-09	Dr. Golding	R. Scotland	Seq. 0016-Draft Labeling(PI)
	Correspondence	24-Dec-09	W. Freas	R. Scotland	BPAC- Briefing Document
	Correspondence	21-Jan-09	W. Freas	R. Scotland	BPAC- Slides
	Correspondence	27-Jan-09	R. Scotland	R. Drews	Labeling Discussion
Seq - 0017	Letter/356h/CD	28-Jan-09	Dr. Golding	R. Scotland	Seq. 0017-Post Approval Commitments
Seq - 0018	Letter/356h/CD	28-Jan-09	Dr. Golding	R. Scotland	Seq. 0018- Lot Release Protocols
	Correspondence	29-Jan-09	User Fees	R. Scotland	User Code

Recombinant Human AT III BLA-125284 Activites

Sequence #	Correspondence	Date	To:	From:	Content
Seq - 0019	Letter/356h/CD	2-Feb-09	Dr. Golding	R.Scotland	Seq-0019
Seq - 0020	Letter /356h/CD	4-Feb-09	Dr. Golding	R. Scotland	Seq. 0020-Draft Labeling
	Correspondence	6-Feb-09	Dr. Epstein	R. Scotland	Approval Letter (Fax)
	Correspondence	6-Feb-09	Dr. Epstein	R. Scotland	Approval Letter -Original